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Role of IL-17 in intestinal immunity against F4⁺ enterotoxigenic *Escherichia coli* infections in pigs

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List of Abbreviations

5-HT	5-hydroxytryptamine
ADP	adenosine diphosphate
AID	activation-induced cytidine deaminase
AIDA	adhesin involved in diffuse adherence
AMPs	antimicrobial peptides
APCs	antigen-presenting cells
APN	aminopeptidase N
APRIL	a proliferation-inducing ligand
BAFF	B-cell activating factor
BD-2	β -defensin 2
cAMP	3',5'-cyclic adenosine monophosphate
CCL2	chemokine (C-C motif) ligand 2
CFAs	colonization factor antigens
CFs	colonization factors
CFTR	cystic fibrosis transmembrane conductance regulator
CgA	chromogranin A
cGKII	cGMP-dependent protein kinase II
cGMP	cyclic guanosine monophosphate
CS	coli surface
CSR	class switch recombination
CT	cholera toxin
CXCL2	chemokine (C-X-C motif) ligand 2
DAEC	diffusely adherent <i>E. coli</i>
DCs	dendritic cells
<i>E. coli</i>	<i>Escherichia coli</i>
EAEC	enteroaggregative <i>E. coli</i>
EAST1	<i>E. coli</i> heat-stable enterotoxin
EatA	ETEC autotransporter A
EDTA	ethylenediaminetetraacetic acid

Abbreviations

EHEC	enterohaemorrhagic <i>E. coli</i>
EIEC	enteroinvasive <i>E. coli</i>
EPEC	enteropathogenic <i>E. coli</i>
ETEC	enterotoxigenic <i>E. coli</i>
F4R	F4 receptor
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
GC	germinal center
GC-C	guanylyl cyclase C
GM-CSF	granulocyte-macrophage colony stimulating factor
GTP	guanosine-5'-triphosphate
HEV	high endothelial venules
IBD	inflammatory bowel disease
IECs	intestinal epithelial cells
ILCs	innate lymphoid cells
ILC3	innate lymphoid cells 3
iNKT	invariant natural killer T
JPP	jejunal Peyer's patches
LT	heat-labile enterotoxin
LTi	lymphoid tissue-inducer
MAPK	mitogen-activated protein kinase
MLN	mesenteric lymph node
MMP-1	matrix metalloproteinase-1
MoDCs	monocyte derived dendritic cells
MR	mannose-resistant
MS	mannose-sensitive
ND	neonatal diarrhea
NF- κ B	nuclear factor- κ B
NK	natural killer cells
PAP	pancreatitis associated protein
pBD-2	porcine beta-defensin 2

Abbreviations

PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCFs	putative colonization factors
PCR	polymerase chain reaction
PGE2	prostaglandin E2
pIgR	polymeric immunoglobulin receptor
PKC	protein kinase C
PMB	polymyxin B
PP	Peyer's patches
PWD	post weaning diarrhea
RPL-19	60S ribosomal protein L19
RT	room temperature
SAA	serum amyloid A
SC	secretory component
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFB	segmented filamentous bacteria
SIgA	secretory IgA
SISP	small intestinal segment perfusion
ST	heat-stable enterotoxins
STEC	shiga-toxin producing <i>E. coli</i>
Stx2e	shiga toxin type 2e
TCR	T cell receptor
TGE	transmissible gastroenteritis
TGF- β	transforming growth factor- β
Th	T helper
TJ	tight junction

Preface

Escherichia coli (*E. coli*) is commonly found in the lower intestine of humans and animals. Most *E. coli* are harmless and even beneficial to the host, for example, boosts digestion through secreting digestive enzymes. However, some *E. coli* are pathogenic causing either diarrhea or extra-intestinal illnesses. Among these pathogenic *E. coli*, enterotoxigenic *E. coli* (ETEC) are one of the most common pathogens causing diarrhea in humans and animals. Indeed, ETEC infections leads to 400 million diarrheal cases and almost 400,000 deaths in young children in ETEC-endemic areas and 20 to 40% traveler's diarrhea cases annually. ETEC is also the common cause of diarrhea and death in young farm animals, including calves, sheep, goat as well as pigs. All over the world, neonatal diarrhea (ND) and post weaning diarrhea (PWD) caused by ETEC result in significant economic losses to the pig production industry due to increased morbidity, mortality, decreased weight gain in survivors, and cost of medication, vaccinations, and feed supplements. This has provided the impetus to find alternative strategies, such as novel vaccines, to prevent PWD in weaner piglets. ETEC infections in neonatal piglets can be prevented with maternal antibodies by vaccination of the sow. However, this passive immunity declines with age and stops at weaning, making the newly weaned piglets highly susceptible to ETEC. To protect newly weaned piglets, extensive studies have been performed to identify the potential immunogen that can protect piglets against challenge infection.

Previous work in our lab demonstrated F4 fimbriae of ETEC are potent mucosal immunogens, since they elicit a fast secretion of F4-specific secretory IgA (SIgA) at the intestinal tissues, protecting piglets against a challenge infection. Recently, T helper type 17 (Th17) cells and their production of IL-17A have been implicated in the induction of SIgA directed against the intestinal microbiota or gut-dwelling pathogens. In order to develop a successful ETEC vaccine, knowledge of the mucosal immune response upon infection is compulsory. Therefore, the following chapters review the present knowledge on F4⁺ ETEC infection in pigs, the virulence factors involved in pathogenesis and the immune responses elicited by the host to combat ETEC infections. In addition, Th17 immunity in both humans and other animals is highlighted.

Chapter 1 Enterotoxigenic *Escherichia coli*

1.1 Introduction

In 1885, *Escherichia coli* (*E. coli*) was discovered by Theodor Escherich when searching for the cause of fatal diarrhea and gastroenteritis. Now, *E. coli* is defined as a gram-negative, facultatively anaerobic bacterium, which is a normal inhabitant of the gastrointestinal tract of humans, endotherms and even reptiles (Berg, 1996; Gordon and Cowling, 2003). As a commensal, the vast majority of the hundreds of *E. coli* strains are harmless or even beneficial, such as producing vitamin K2 (Bentley and Meganathan, 1982) and regulating intestinal inflammation (Henker et al., 2008; Schultz, 2008). However, *E. coli* can also cause enteritis, urinary tract infection, septicemia and other clinical infections (Allocati et al., 2013). These pathogenic *E. coli* are classified into several groups based on their virulence factors and the clinical symptoms they trigger in the host (Nataro and Kaper, 1998; Kaper et al., 2004): enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC) or shiga-toxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enteropathogenic *E. coli* (EPEC) as shown in **Table 1**.

Table 1. *E. coli* pathogenic types

Enteropathogenic <i>E. coli</i>	Diseases	Symptoms	Virulence factors
Enterotoxigenic <i>E. coli</i> (ETEC)	Traveler's Diarrhea	Watery diarrhoea and vomiting	CFs, LT and ST
Enterohaemorrhagic <i>E. coli</i> (EHEC)	Haemorrhagic colitis, HUS	Bloody diarrhoea	Shiga toxins, Intimin, Bfp
Enteroinvasive <i>E. coli</i> (EIEC)	Shigellosis-like	Watery diarrhoea; dysentery	Shigatoxin, hemolysin, Cellular invasion, lpa
Enteroaggregative <i>E. coli</i> (EAEC)	Diarrhoea in children	Diarrhoea with mucus and vomiting	AAFs, cytotoxins
Adherent invasive <i>E. coli</i> (AIEC)	Associated with Crohn disease	Persistent intestinal inflammation	Type 1 fimbriae, Cellular invasion
Diffusely adherent <i>E. coli</i> (DAEC)	Acute diarrhoea in children	Watery diarrhoea, recurring UTI	Daa, AIDA
Enteropathogenic <i>E. coli</i> (EPEC)	Diarrhoea in children	Watery diarrhoea and vomiting	Bfp, Intimin, LEE

HUS: haemolytic-uraemic syndrome; Bfp: Bundle-forming pili; lpa: Invasion plasmid antigen; AAF: aggregative adherence fimbria; Daa: diffuse adhesin; AIDA: adhesion involved in diffuse adherence; LEE: Locus for enterocyte effacement.

Among these pathogenic *E. coli*, ETEC are the most common pathogens causing diarrhea in humans, leading to approximately 400 million diarrheal cases and almost 400,000 deaths per year in children aged less than 5 years in ETEC-endemic areas (Qadri et al., 2005; WHO, 2009). ETEC infection is also very common in livestock species, especially in pigs and cattle. In neonatal and recently weaned piglets, ETEC infections typically cause a severe watery diarrhea, resulting in financial losses to the swine industry (Nagy and Fekete, 1999). ETEC, together with other microbial pathogens, including rotavirus, *Salmonella spp*, transmissible gastroenteritis (TGE) virus, *Lawsonia intracellularis*, *Clostridium perfringens* and coccidiosis, have been associated with diarrhea in piglets. Notably, ETEC is the most common cause of diarrhea in neonatal and newly weaned piglets (Chen et al., 2004; Katsuda et al., 2006; Kim et al., 2010b; Chan et al., 2013).

1.2 Transmission of ETEC

For animals, including pigs and calves, ingestion of contaminated fecal and water is the main route of ETEC infection. Newborn piglets can be easily exposed to ETEC from the mammary glands of the sow and the farrowing crates or pens with ETEC diarrhea (Gyles, 2010). The environment of the weaner unit appears to be the most likely source of pathogenic *E. coli* for the newly weaned piglets and previously contaminated farrowing crates or their dam are considered to be the contagious agent for the suckling pigs (Jeffrey et al, 2012). In addition, caretakers can also transmit ETEC to susceptible pigs during direct contact (Amass et al., 2003). Notably, the bacteria can be also transmitted to other pigs via contaminated aerosols (Cornick and Vukhac, 2008; Duan et al., 2009). Moreover, ambient temperatures in the farrowing house may also influence the transmission of the bacteria, probably by reducing the activity of intestinal peristaltic activity and passage of bacteria and protective antibodies (Sarmiento, 1983). Increased numbers of pathogenic *E. coli* in the intestinal tract of these pigs result in a more severe diarrhea in pigs kept at 30°C as compared to pigs housed at below 25°C (Gyles, 2010). A mathematical model for *E. coli* infection dynamics in cattle also confirmed this finding and suggests more transmission can occur at higher temperatures in summer (Wang et al., 2014).

After ingestion, ETEC have to pass through the stomach before they can colonize the lower part of the intestine. Thus, the bacteria first need to survive a wide pH range and harsh environmental conditions. In adult pigs, acidic conditions in the stomach form an efficient barrier against *E. coli* and other pathogens (Zhu et al., 2006). In weaned pigs, however, the less acidic environment in the stomach and duodenum and the lower production of digestive enzymes, enhances survival of ETEC (Jensen, 1998; Franklin et al., 2002). Once ETEC pass through the stomach, they bind to the receptors on the small intestinal epithelium or in the mucus coating the epithelium. These bacteria then reproduce rapidly, typically attaining 10^9 CFU/g in the mid-jejunum and ileum (Gyles, 2010). When the colonization is established, ETEC secrete enterotoxins, which stimulate the secretion of water and electrolytes, resulting in a watery diarrhea (**Figure 1**). Fluid loss may lead to dehydration, depression, metabolic acidosis and death. ETEC infection can also significantly increase paracellular permeability of the small intestine through the modulation of tight junctions (TJ) (Berkes et al., 2003; Moretó and Pérez- Bosque, 2009). The increased permeability of the epithelial barrier due to ETEC infection further increases transport of foreign molecules, toxins or even pathogens and can trigger inflammatory cascades (Berkes et al., 2003). ETEC are also described to degrade mucins in the small intestine and as such promote bacterial colonization of the small intestine and accelerate toxin access to the enterocyte surface (Kumar et al., 2014; Luo et al., 2014). ETEC infection therefore can cause hemorrhagic gastroenteritis, microvascular fibrinous thrombi and villi necrosis (Fairbrother et al., 2005).

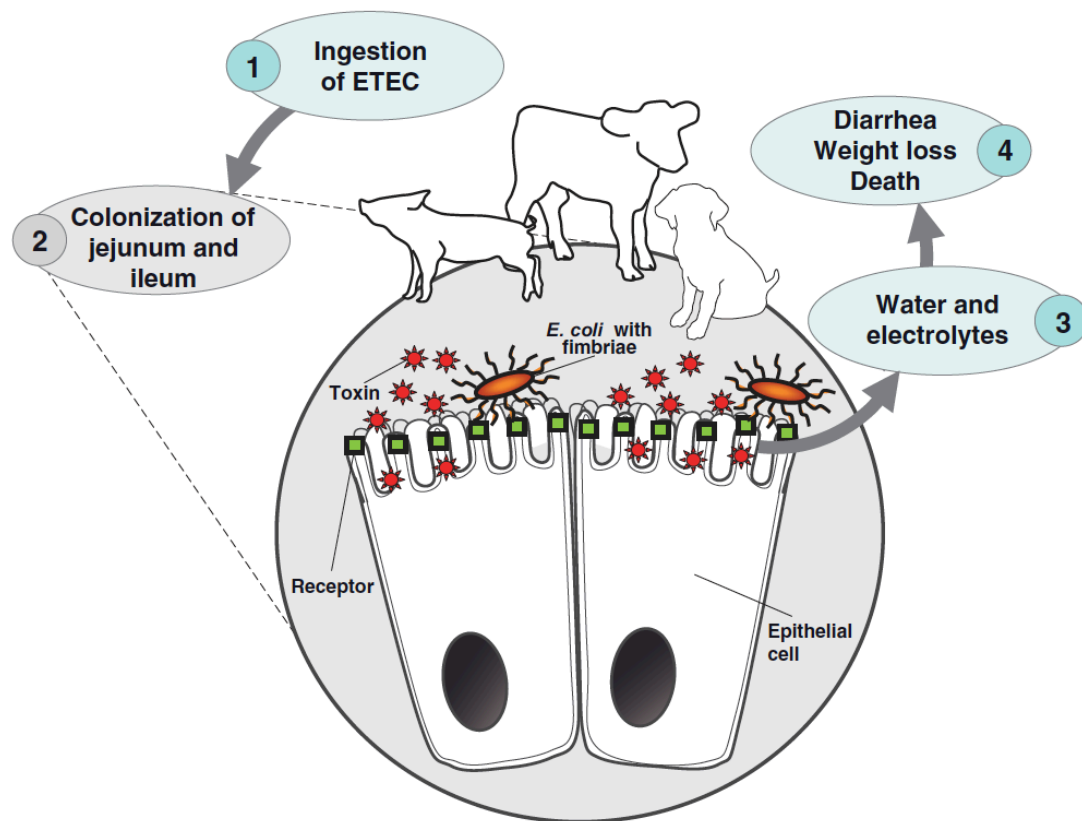


Figure 1. Schematic representation of the steps involved in the pathogenesis of an ETEC infection. ETEC acquired by ingestion and (1), pass through the stomach, adhere to the small intestinal epithelium where they produce enterotoxins (2). These enterotoxins stimulate the secretion of water and electrolytes into the intestinal lumen (3). Loss of water and electrolytes leads to diarrhea, weight loss, and possibly death (4). Reviewed by Gyles and Fairbrother, 2010. Reference Laboratory for *Escherichia coli*, Faculty of Veterinary Medicine, Université de Montréal.

1.3 Porcine ETEC strains

1.3.1 Serotypes of porcine ETEC strains

The classification of different ETEC strains started with the identification of surface antigens O (somatic) and H (flagellar) on the bacteria and their flagella. More than 700 serotypes of *E. coli* have been identified using a combination of O and H antigens (Eisenstein et al, 2000; Griffin et al., 1991), and possibly more O:H combinations will be discovered (Robins-Browne and Hartland, 2002). Via bacterial agglutination tests *E. coli* also used to be serotyped by their K (capsular) antigens (Nataro and Kaper, 1998). Since several different molecular structures, including fimbriae, conferred the K phenotype, proteinaceous fimbrial (F) antigens have therefore been introduced and replaced the K series (Orskov et al., 1982). Due to the limited sensitivity and specificity, and the various combinations of antigens, serotyping is tedious and expensive and is performed reliably only by a small number of reference laboratories (Stenutz et al., 2006). However, several important O antigens in ETEC infected piglets were identified in many clinical laboratories by polymerase chain reaction (PCR)-based methods to detect the presence of specific O and H antigen genes (Prager et al., 2003). Although several O serotypes, including O8, O9, O101, O149 and O157, have been commonly isolated in cases of neonatal diarrhea in different countries, the O149 serogroup has been determined to be the dominant serogroup in cases of neonatal and PWD caused by ETEC in many countries (Chen et al., 2004; Fairbrother et al., 2005; Frydendahl, 2002; Vu-Khac et al., 2007; Gyles, 2010; Byun et al., 2013a). In addition, certain serotypes correlated with the presence of specific fimbrial types, which will be further discussed in next sections.

1.3.2 Colonization factors and fimbriae subtype

ETEC strains adhere to the small intestinal mucosa via colonization factors (CFs), including colonization factor antigens (CFAs), putative colonization factors (PCFs) and coli surface (CS) antigens (Gaastra and Svennerholm, 1996; Deshpande, 2002). Currently, at least 26 CFs have been recognized among human ETEC strains, which are mainly fimbrial or fimbriar proteins (Fleckenstein et al., 2013). Since the

determination of the ETEC serotype is very complex, fimbrial subtypes are usually used to distinguish ETEC isolates. ETEC strains causing disease in animals use unique fimbriae, which are in general not found in human ETEC strains. For example, porcine-specific ETEC isolates express any of five fimbriae: F4 (K88), F5 (K99), F6 (987P), F7 (F41) and F18, while bovine and ovine ETEC strains synthesize F5 and F7 (Blanco et al., 1991; Nagy and Fekete, 1999). Epidemiological data reveal that the frequencies of fimbrial types display geographical differences (summarized in **table 2**). Among these five different fimbrial subtypes, F4 fimbriae are the most frequently associated with ETEC-induced diarrhea in piglets in countries such as Belgium, Slovakia, USA, Italy and Australia (Van den Broeck et al., 1999c; Vu-Khac et al., 2004, 2007; Zhang et al., 2007; Luppi et al., 2014; Do et al., 2005). Comparatively, F18 fimbriae are dominant in some areas including Cuba, South Korea and China (Blanco et al., 2006; Kim et al., 2010b; Chen et al., 2004; Cheng et al., 2006). ETEC strains expressing F4 or F18 fimbriae are found on ETEC that predominantly cause PWD, while F5 and F6 fimbriae are rarely isolated from diarrheal piglets (Kwon et al., 1999, 2002; Vu-Khac et al., 2004) (summarized in **table 2**). Although F7 fimbriae are usually present in combination with F5 fimbriae, in some areas they are the most prevalent adhesin. Intriguingly, in Mexico F4 and F18 fimbriae are less frequent and are in fact rarely isolated from diarrheic piglets (Toledo et al., 2012). Serotype O149 is the most prevalent serotype in neonatal and 1-3 week old piglets, while serotype O149 is the most prevalent serotype in weaned piglets. Importantly, serotype O149 and O141 are always associated with the presence of F4 and F18 fimbriae in ETEC isolated from diarrheic piglets (Byun et al., 2013a; Vu-Khac et al., 2007; Do et al., 2005).

Table 2. Prevalence and fimbrial genotype distribution of ETEC isolated from diarrheic piglets

Country	Prevalence in PWD cases (%)							Age of pigs	Reference
	F4 (K88)	F18	F5 (K99)	F6 (987P)	F7 (F41)	F17	intimin		
USA	64.6	34.3	0.57	0	0.57	not tested	not tested	Post weaned, 4-6 week-old, 175 isolates	Zhang et al., 2007
	19	35	0.9	5	0.9	3	0.9	Post weaned, 101 isolates	Vu-Khac et al., 2006
Slovakia	23.9	10.8	F5+F7=1.1	3.2	F5+F7=1.1	not tested	not tested	Unweaned, 2-4 week-old, 92 isolates	Vu-Khac et al., 2004
	38	9	F5+F7=3	3	F5+F7=3	1.4		<2 weeks old, 220 isolates	Vu-Khac et al., 2007
Italy	77.3	22.3						28 to 45 days old, 475 isolates	Luppi et al., 2014
Australia (Queensland)	42.5	56.9	31.3	not tested	0	not tested	not tested	neonatal and weaned pigs, 52 isolates	Chapman et al., 2006
	66.7	0	6.7	6.7	not tested	not tested	not tested	neonatal, 1 and 5 days of age, 15 isolates	
	60.9	0	0	0	not tested	not tested	not tested	4-6 week-old, 23 isolates	Do et al., 2005
	65.9	10.2	not tested	not tested	not tested	not tested	not tested	post weaned, 88 isolates	
China (Eastern)	14.8	31.5	4.6	12.0	3.7	not tested	not tested	post weaned, 4-10 weeks, 108	Chen et al., 2004
China (Jiangsu Province)	3.8	26.3						weaned piglets with PWD and/or ED	Cheng et al., 2006
Brazil (Londrina)	44	38	30	25	32	not tested	not tested		Vidotto et al., 2009
Canada	44.7	39.3	not tested	0.9	not tested	not tested	not tested	4-8-week-old pigs, 219 isolates	Frydendahl et al., 2002
Vietnam	53.1	0	16.7	0	0	not tested	not tested	preweaning piglets in commercial piggeries	Do et al., 2006a
Serbia	39.86							1-30 days	Jadranka et al., 2010
South Korea	7.45	15.43	29.79	6.38	3.72			suckling and weaning periods	Lee et al., 2008
Cuba	0	61	0	8	0	not tested	not tested	young piglets<30 days and post weaned >30 days	Blanco et al., 2006
Cuba (Villa Clara province)	23	19	4	4	4	not tested	not tested	2 to 25days and 26 to 48 days, 26 isolates	de la Fé Rodríguez et al., 2013
Hungary	61	not tested	not tested	not tested	not tested	not tested	not tested	4-10 weeks age, PWD, 205 isolates	Nagy et al., 1990
Denmark	44.7	39.3	not tested	0.9	not tested	not tested	not tested	4-8-week-old pigs with diarrhoea or ED, 219 <i>E. coli</i> isolates	Frydendahl, 2002
Mexico	3	9	8	12	72	9	not tested	0-3 weekd, and 3-5 weeks age. 953 <i>E. coli</i> isolates	Toledo et al., 2012

1.4 Virulence factors of porcine ETEC strains

1.4.1 Fimbriae

ETEC strains from both humans and animals adhere to small intestinal mucosa via CFs, which are mainly fimbrial proteins. Fimbriae are filamentous protein appendages, which are peritrichously distributed in numbers of 100-300 or even up to 1000 per bacterium (Van den Broeck et al., 2000). These fimbriae are relatively host specific. For example, porcine ETEC strain expressing F4 (K88) cause disease only in pigs, while F5 (K99)-containing strains cause disease in piglets, calves and lambs (Blanco et al., 1991; Nagy et al., 1999). In this section, only F4 and F18 fimbriae will be discussed in detail since they are the most prevalent in ETEC-associated diarrheal piglets.

Based on their hemagglutinating properties, fimbriae are classified as mannose-sensitive (MS) and mannose-resistant (MR). F4 and F18 fimbriae both belong to the MR fimbriae and are 2-4 nm in diameter, while their length varies between 0.2 and 20 μ m (Krogfelt, 1991). F4 fimbriae are encoded by the *fae* locus on a plasmid with a molecular weight ranging from 50 to 177 MDa (reviewed in Verdonck, 2004c) and the corresponding subunits were nominated FaeA through FaeJ (Van den Broeck et al., 2000). The *fae* gene cluster or the F4 operon encodes the regulatory proteins FaeA and FaeB, the major subunit FaeG, the minor subunits FaeC, FaeF, FaeH and FaeI, the usher FaeD and the chaperone FaeE (Van den Broeck et al., 2000). Interestingly, ETEC fimbriae and toxins encoding genes are often present on the same plasmid (reviewed in Verdonck, 2004c).

As shown in **Figure 2**, F4 fimbriae are mainly composed of the major subunit FaeG with low amounts of FaeC, FaeF and FaeH (Van den Broeck et al., 2000). Unlike most fimbriae, which have tip-localized adhesin minor subunits, the major structural subunit FaeG is the adhesin of F4 fimbriae responsible for binding to intestinal receptors (reviewed in Vipin Madhavan and Sakellaris, 2015). During F4 fimbriae assembly, the usher FaeD translocates the fimbrial subunits (**Figure 2**)

through the outer membrane to allow their assembly in the fimbrial shaft on the bacterial membrane (Mooi et al., 1986). Although not very clear, the minor subunit FaeC probably initiates the process of F4 fimbrial synthesis (**Figure 2a**), since this subunit is particularly present at the tip of the F4 fimbriae (Oudega et al., 1989). Subsequently, FaeF functions as an adaptor molecule, coupling the major subunit FaeG to the tip subunit FaeC (**Figure 2b,c**). Similarly, the FaeH subunit is probably responsible for the elongation of the fimbriae (**Figure 2d,e,f**), since both FaeF and FaeH proteins are found at regular distances along the fimbrial shaft (Bakker et al., 1992a). These two subunits also play a key role in the initiation of fimbrial assembly, since mutants lacking either the FaeF or FaeH polypeptide are impaired in the production of fimbriae (reviewed in Van den Broeck et al., 2000). The chaperone FaeE, which is located in the periplasm, assists in the folding of the FaeG and FaeH subunits into an export-competent configuration and transports them to the outer membrane where they are incorporated in the growing fimbriae. Moreover, such folded complexes are protected against proteolytic degradation and premature polymerization (Bakker et al., 1991). After transportation, the chaperone FaeE recycles in the periplasm to transport another subunit. Remarkably, FaeE cannot form a complex with FaeF, indicating other mechanisms may exist to transport fimbrial subunits to the outer membrane (Mooi et al., 1982). Considering the structural similarities with FaeF, FaeC probably does not form complexes with FaeE either (Mol et al., 1995). Considering FaeI and FaeJ share homology with FaeH, it is likely that FaeI and FaeJ are also minor fimbrial subunits, but no studies confirmed their presence in or along the fimbrial structure (Bakker et al., 1992b). Thus, more research should be performed to elucidate the function of the other subunits, especially FaeA, B, I and J, during the biosynthesis of F4 fimbriae.

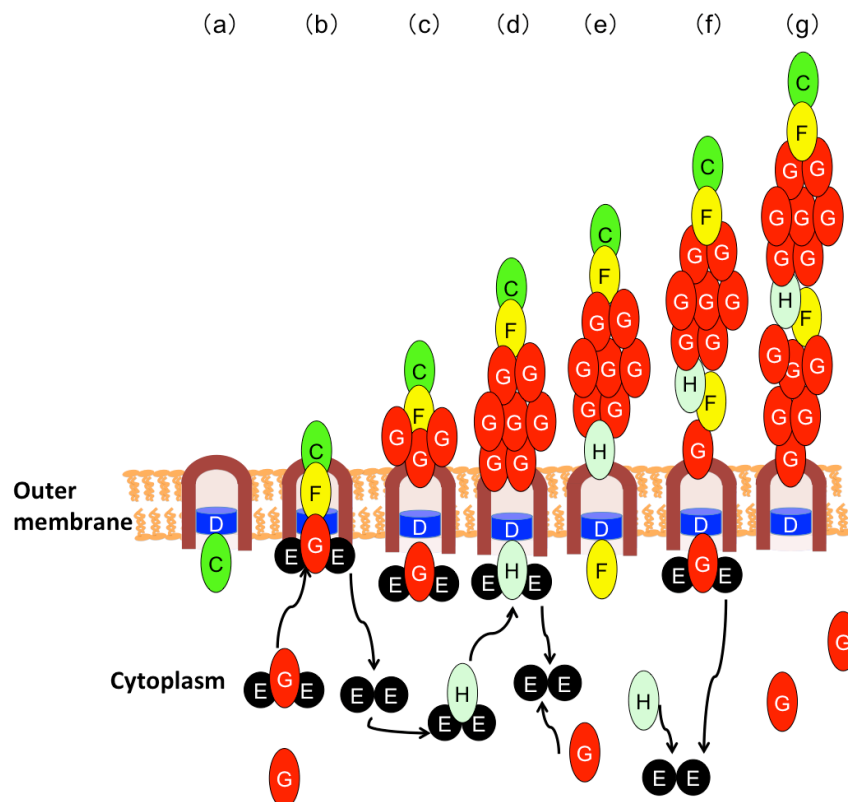


Figure 2. F4 fimbriae biosynthesis model. The usher FaeD is involved in the translocation of the fimbrial subunits across the outer membrane and is anchored in the outer membrane. FaeC and FaeD initiate the F4 formation, while the chaperone FaeE assists the FaeG and FaeH subunits to FaeD, to allow their incorporation in the growing fimbrial structure. Adapted from Van den Broeck et al., 2000. Adapted from Iwakura et al., 2011. Reuse with permission from Elsevier Publishers Ltd: License Number: 3765390828399

Epitope analysis of the F4 fimbriae revealed the existence of at least 11 epitope clusters on the F4 antigen complex designated a1 to a7, b1, b2, c, and d. The “a” stands for a common epitope, whereas “b”, “c” and “d” represent specific epitopes (van Zijderveld et al., 1990). Thus, three antigenic variants of F4 fimbriae were identified, referred to as F4ab, F4ac and F4ad, which differ in the primary sequence of FaeG. These variants displayed a related, but different binding and hemagglutination profile (Guinée and Jansen, 1979). Among these three F4 fimbrial variants, the F4ac fimbriae are the most prevalent type detected throughout the world (Choi and Chae, 1999; Holoda et al., 2005; Byun et al., 2012).

Its binding place in the brush border of small intestinal enterocytes has been termed as F4 receptor (F4R) (Van den Broeck et al., 2000). In the 1970s, several pioneers initiated the identification of intestinal receptors for F4 fimbriae and found that F4⁺ ETEC only bind to intestinal cells of susceptible piglets, but not adult pigs or humans (Gibbons et al., 1975; Sellwood et al., 1975). It is now generally accepted that these F4R are a variety of glycoproteins and glycolipids, which have been identified from the brush borders of epithelial cells, intestinal membranes and mucus (Jin and Zhao, 2000; Van den Broeck et al., 2000). However, the three different variants of F4 (F4ab F4ac and F4ad) have their own specific receptors (Billey et al., 1998). This binding ability of F4 variants was confirmed by a chromatogram binding assay and indicated that glycosphingolipids may also be the receptors/ligands of porcine F4⁺ ETEC (Coddens et al., 2011). Aminopeptidase N (APN) was recently identified as an epithelial receptor for F4⁺ ETEC, which promotes internalization of F4ac fimbriae through clathrin-mediated endocytosis (Melkebeek et al., 2012). The F4R on the brush border can be inherited in a dominant way and generally independent of age. Indeed, F4R is expressed on enterocytes throughout the piglets entire life time, although this expression decreases between 3 and 8 weeks of age, making older pigs less susceptible to F4⁺ ETEC infection (Chandler et al., 1994; Baker et al., 1997). Interestingly, the F4R is also found in intestinal mucus, probably due to enterocyte turnover, and their function is to bind F4⁺ ETEC and facilitate the removal of bacteria from the intestine (Conway et al., 1990). Thus, F4R in the mucus could help to protect piglets from ETEC infection. Similarly, the presence of F4R in the mucus declines significantly at the time of weaning and ceases by 6 months of age (Chandler et al., 1994). Moreover, the distribution of the F4R varies along the porcine intestinal tract. The amount of F4R was found to be highest in mucosal villi taken from the mid-small intestine using an enzyme immunoassay. Indeed, *in vitro* assays demonstrated that all three F4 variant strains displayed much higher adhering capacity to jejunal than to duodenal villi of susceptible piglets (Cox and Houvenaghel, 1993). This result was further confirmed by an enzyme immunoassay (Chandler et al., 1994). Even though many studies have been carried out to identify the F4R, no two groups reported the

same molecular weights, indicating the complexity of the binding place in the intestine for F4⁺ ETEC adherence.

F18 fimbriae are encoded by the *fed* gene cluster, which is composed of five genes: *fedA*, *fedB*, *fedC*, *fedE* and *fedF* (Smeds et al., 2001). These genes together with genes coding for the haemolysin and enterotoxin are located on plasmids of varying size (42-98 MDa) (reviewed in Smeds, 2003; Johnson and Nolan, 2009). Besides, the gene encoding adhesin involved in diffuse adherence (AIDA), an afimbrial adhesin, is also frequently found on the same plasmid as the *fed* gene cluster (Mainil et al., 2002). The full F18 fimbrial structure consists of the major subunit FedA, two associated minor subunits FedE and FedF, a putative usher protein FedB and the chaperone FedC (Imberechts et al., 1992, 1996; Smeds et al., 2001). Unlike F4 fimbriae, the minor subunit FedF, which is located at the tip of the fimbriae, is essential for F18⁺ ETEC adhesion (Smeds et al., 2001). The role of FedE is not clear, although it is presumably has a role as molecular ruler as its deficiency affects fimbrial length (Imberechts et al., 1996; Smeds et al., 2003). Indeed, FedE- and FedF-deficient mutants produce significantly longer fimbriae than the wild F18 fimbriae strain (Imberechts et al., 1996), indicating their regulatory roles in fimbrial biosynthesis. Like F4 fimbriae, F18 fimbriae also have two antigenic variants, termed F18ab and F18ac (Rippinger et al., 1995; Nagy et al., 1992; Salajka et al., 1992). F18ac is the dominant subtype in ETEC-linked diarrheal piglets and mainly cause PWD, while F18ab are more often associated with Stx2e producing STEC strains and causes edema disease (Cheng et al., 2005; Byun et al., 2013b; Barth et al., 2011). Recently, new F18 variants have been detected, which belonged to O121, O101 and O146 serotypes (Barth et al., 2011; Byun et al., 2013b). These serotypes are rarely found in common F18 ETEC strains (**Table 3**).

Table 3. Virulence factors of porcine-specific ETEC strains and associated disease.

Adhesins	Toxins	O Serogroups	Disease
F5, F6, F7	STa	O8, O9, O20, O64, O101	Neonatal diarrhea
F4	STa, STb, LT, EAST-1, α -hemolysin	O8, O138, O141, O145, O147, O149, O157	Neonatal diarrhea, diarrhea in young pigs preweaning
F4, AIDA, unknown	STa, STb, LT, EAST-2, α -hemolysin	O8, O138, O139, O141, O147, O149, O157, O?:K48	PWD
F18, AIDA	STa, STb, LT, Stx (VT), EAST-1, α -hemolysin	O8, O138, O139, O141, O147, O149, O157	PWD
F18	Stx2e	O138, O139	Edema disease

1.4.2 Heat-labile enterotoxin (LT)

Once colonization is established, ETEC produce enterotoxins, including the heat-labile enterotoxin (LT) and/or heat-stable enterotoxins (ST) that act upon intestinal enterocytes causing secretory diarrhea. LT was originally named to distinguish the heat stability of enterotoxigenic factors, since LT can be inactivated at 70°C in 10 minutes, whereas ST enterotoxins are still active upon boiling (Gill et al., 1981). LT comes in two variants, LT-I and LT-II. The former one is a multimeric AB₅ toxin, composed of a single A subunit (LTA) associated with a ring of five B subunits (LTB) (**Figure 3A**) (Hardy et al., 1988). LT-I comprises two closely related antigenic variants, called LTp-I and LTh-I, which are isolated from pig and human ETEC strains, respectively (Tsuji et al., 1982). The A subunit of LTp-I and LTh-I has a similar size (Geary et al., 1982), but differs in three amino acids (AA). Likewise, their B subunits also differ in three or more AA depending on the ETEC strains (Kazemi and Finkelstein, 1990). Because of the high homology of the AA sequence, LTp-I and LTh-I share some antigenic determinants and display related, but not identical immunological properties (Honda et al., 1981). This AA difference in the B subunit of LTp-I and LTh-I also seems to result in a different receptor binding ability (Karlsson et al., 1996). For example, these two toxins bind to glycosphingolipid receptors (e.g., GM₁ ganglioside) and polyglycosylceramides, but only LTp-I is able to bind paragloboside.

LT-I resembles cholera toxin (CT) in its structure and function, in which the B subunit is responsible for binding to glycosphingolipid receptors on the surface of eukaryotic cells (e.g., GM₁ ganglioside). Binding of LT-I to its receptor results in internalization into the cells. Once internalized, the A subunit of LT-I covalently modifies the α subunit of the Gs guanosine-5'-triphosphate (GTP)-binding protein via its adenosine diphosphate (ADP)-ribosylation activity, resulting in the constitutive activation of adenylate cyclase and the production of 3',5'-cyclic adenosine monophosphate (cAMP) (Spangler, 1992). Increased levels of cAMP lead to activation of the cAMP-dependent protein kinase A (PKA), which phosphorylates the R domain of the cystic fibrosis transmembrane conductance regulator (CFTR) (Viswanathan et al. 2009). These phosphorylated CFTR channels remain open and lead to the secretion of chloride ions (Cl⁻) and HCO₃⁻ ions from secretory epithelial cells into the lumen. In addition, the absorption of sodium ions (Na⁺) is also decreased, since they bind to the negative chloride ions. Increased cAMP production may also inhibit Na⁺ influx into the enterocytes (Hyun and Kimmich, 1982). Subsequently, water follows by osmotic drag and excessive secretion of electrolytes and water leads to dehydration, metabolic acidosis, and even death. On top of its effect on ion pumps, cAMP accumulation also downregulates the secretion of antimicrobial peptides by the intestinal epithelial cells (Chakraborty et al., 2008), which may bring pathogens into closer contact with the epithelial cells. Both *in vitro* and *in vivo* experiments indicated LTp-I has less toxicity as compared to LTh-I (Lasaro et al., 2008), probably because porcine LT-I is less efficient in activating ADP ribosylation (Rodrigues et al., 2011). However, ETEC constructs expressing porcine or human LT-I are equivalently virulent in causing diarrhea in gnotobiotic pigs (Zhang et al., 2008). LT-I significantly contributes to diarrhea in neonatal gnotobiotic pigs, since 60% of piglets inoculated with an LT-I-negative mutant developed severe dehydrating diarrhea and septicemia as compared to 100% of animals inoculated with the parent LT-positive strain (Berberov et al., 2004; Zhang et al., 2006). LT-I is not only involved in the initiation of diarrhea, but also seems to promote adherence of ETEC *in vitro* and *in vivo* (Berberov et al., 2004; Johnson et al., 2009a). This LT-promoted ETEC adherence was linked to LT-dependent activation of the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways

(Wang et al., 2012). Elevated LT-I production in ETEC was found in the environment with pH values above 7 and a maximal secretion of LT-I was observed at pH 8.6 (Hegde et al., 2009; Gonzales et al., 2013). However, LT-I secretion was completely inhibited at pH 5 (Gonzales et al., 2013). Considering the pH in newly weaned piglets ranges between 2.6-4.9 and 4.5-7.3 in the stomach and small intestine (reviwd in Maré, 2009), respectively, this mechanism of LT-I secretion may help the bacteria to sense the pH gradient and colonize the appropriate part of small intestine.

LT-II is also an AB₅ toxin and its A subunit has about 57% similarity with the A subunit of LT-I and CT, whereas the B subunit of LT-II does not show any significant similarity to LT-I (Nataro and Kaper, 1998). Correspondingly, LT-II is antigenically distinct from LT-I and cannot be neutralized by anti-LT-I or anti-CT antisera. LT-I variants encoded by plasmids in ETEC from humans and pigs have amino acid sequences that are $\geq 95\%$ identical. In contrast, LT-II toxins are chromosomally encoded and are much more diverse from different species. LT-II expressing ETEC strains are rarely isolated from human patients, but seem to be associated with disease in calves (Salvadori et al., 2003; Rigobelo et al., 2006; Jobling and Holmes, 2012). In line with their amino acid similarity, CT and LT-I bind strongly to ganglioside GM1, while LT-II binds to other gangliosides, such as ganglioside GD1a and GD1b (Fukuta et al., 1988). It is thought that this different ganglioside-binding activity might contribute to the host specificity and intoxication particular animal species, tissues or cell types (Connell et al., 2007). Until now, there is no evidence that LT-II is associated with diarrhea in piglets.

1.4.3 Heat-stable enterotoxin (ST)

In contrast to LT (84,000 Da), ST is a small polypeptide (2,000 Da), which is nonimmunogenic in its natural form. ST has two major genotypes, STa and STb, both causing diarrhea. STa (also called STI) is an 18- or 19-amino acid cysteine-rich peptide, which is encoded by the *estA* gene with two different subtypes STaP (or STIa) and STaH (or STIb) (Rao, 1985). STaP is mainly found in porcine and bovine ETEC strains, whereas STaH is only produced by human ETEC strains (Gyles, 2010). In contrast,

STb (or STII) is predominantly produced by animal ETEC strains. However, the presence of these enterotoxins varies in isolated ETEC strains (Qadri et al., 2005). Upon colonization, ETEC produces the STa enterotoxins and bind to a guanylyl cyclase C (GC-C) glycoprotein receptor on the brush border of villous and crypt intestinal epithelial cells. This interaction activates the guanylate cyclase and results in the production of cyclic guanosine monophosphate (cGMP) (Al-Majali et al., 2007). Elevated levels of intracellular cGMP activate cGMP-dependent protein kinase II (cGKII), resulting in activation of CFTR (Gyles, 2010, **Figure 3B**). As mentioned above, activation of CFTR results in secretion of Cl^- and HCO_3^- as well as inhibiting the uptake of Na^+ . Compared to LT, STa seems to induce fluid secretion more rapidly in the intestine (Zhang et al., 2008; Read et al., 2014). In addition, a combination of STa and LT induced a greater fluid accumulation in mice than those exposed to either enterotoxin alone, especially early after exposure. This synergistic effect could be partially explained by the observation that LT increases the levels of cGMP induced by STa in epithelial cells, although the cAMP production triggered by LT did not change in the presence of STa (Read et al., 2014). Besides triggering secretion, STa is also involved in actin cytoskeleton rearrangements (Mahata et al., 2010), which may mediate bacterial internalization. Interestingly, STa and analogues can inhibit the proliferation of colon cancer cells, which could be explained by the ability of STa to delay the progression of the cell cycle in these cells in a cGMP- and Ca^{2+} -dependent pathway (Saha et al., 2008; Giblin et al., 2006; Weiglmeier et al., 2010).

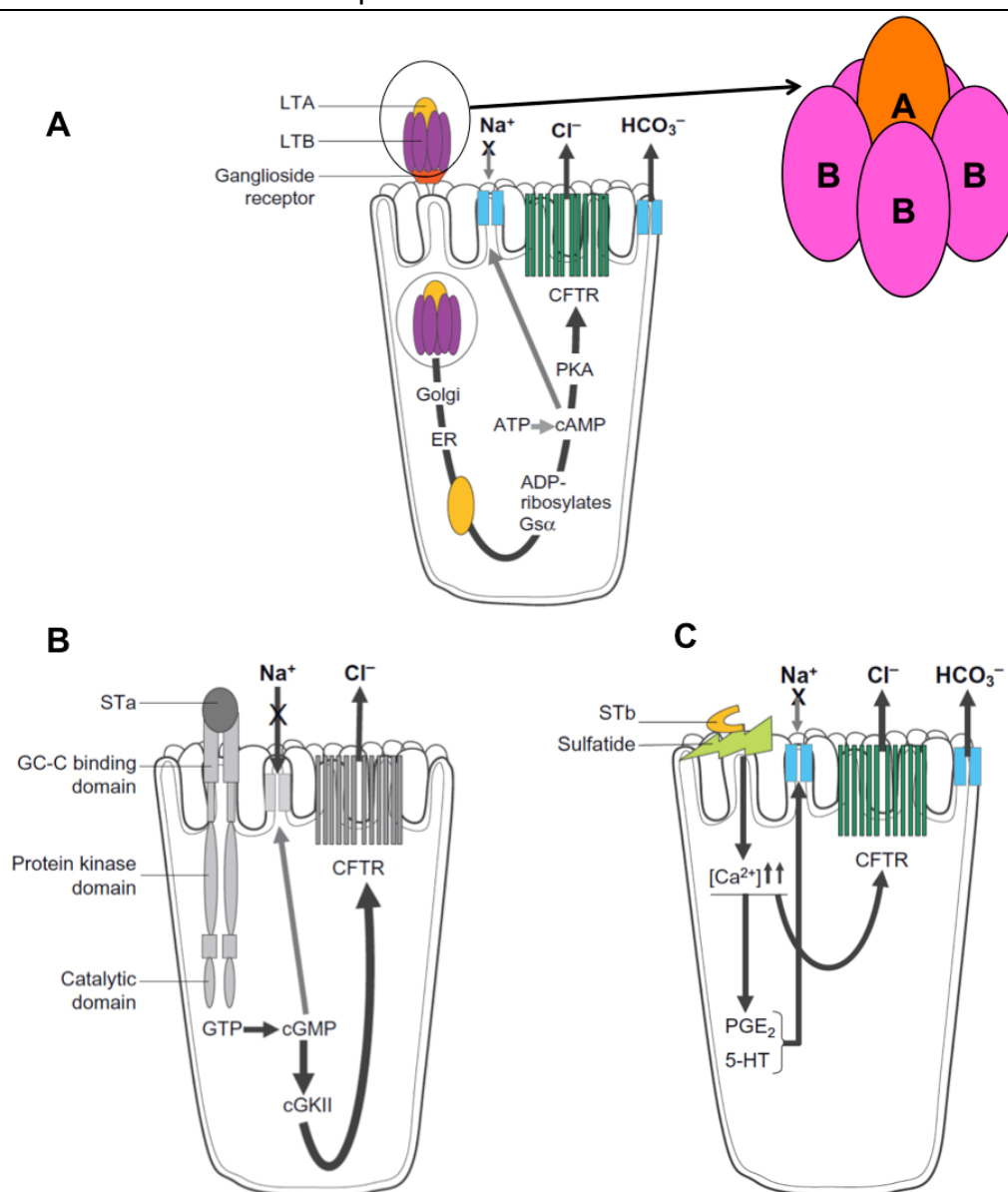


Figure 3 Major mechanism of action of the ETEC enterotoxins LT and ST. A) Mechanism of action of LT: LT binds GM₁ and other gangliosides, resulting in internalization by receptor-mediated endocytosis. Following transport to the Golgi and the endoplasmic reticulum, LTA is translocated to the cytosol where it ADP-ribosylates the regulatory protein Gsα, which irreversibly stimulates production of cyclic AMP. Elevated levels of cAMP increase production of cAMP-dependent protein kinase A, leading to phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR activation leads to secretion of Cl⁻ from secretory epithelial cells in the crypts, which stimulates HCO₃⁻ secretion from the Cl⁻/HCO₃⁻ exchanger in villus epithelial cells. In addition, elevated cAMP levels prevent absorption of Na⁺. **B) Mechanism of action of STa:** Binding of STa to its receptor (GC-C) activates the catalytic domain, causing conversion of GTP to cGMP, which will activate the cGMP-dependent kinase cGKII. This kinase phosphorylates CFTR, causing secretion of Cl⁻ and HCO₃⁻ and inhibition of Na⁺ absorption. **C) Mechanism of action of STb:** Binding of STb to its receptor sulfatide leads to elevated intracellular levels of calcium. The high calcium level activates the CFTR and induces the production of the secretagogues, prostaglandin E₂ (PGE₂) and 5-hydroxytryptamine (5-HT). These trigger the secretion of Cl⁻ and HCO₃⁻ and also inhibit Na⁺ absorption. Reference Laboratory for *Escherichia coli*, Faculty of Veterinary Medicine, Université de Montréal.

STb is a 48 amino acid peptide, mostly associated with porcine and bovine ETEC isolates, but it has also been reported in human isolates (Lortie et al., 1991; Okamoto et al., 1993). Unlike LT or STa, STb stimulates intestinal secretion independent of cGMP or cAMP production (Hitotsubashi et al., 1992). Instead, STb binds to its intestinal epithelial cell receptor sulfatide (Beausoleil et al., 1999; Gonçalves et al., 2008). Sulfatide is a 3-O-sulfogalactosylceramide that is synthesized from ceramide by two transferases, ceramide galactosyltransferase and cerebroside sulfotransferase (Takahashi and Suzuki, 2012). The activity of STb in the gut of rats and mice is considered to be limited, since trypsin (and trypsin-like) proteases suppresses its activity (Whipp, 1987). However, STb in the pig gut still possess its toxin activity, eliciting a very fast fluid response (Hitotsubashi et al., 1992; Loos et al., 2012). STb was shown to increase intracellular levels of Ca^{2+} (Dreyfus et al., 1993), which stimulates protein kinase C (PKC)-mediated CFTR activation (**Figure 3C**). In addition, the increased Ca^{2+} levels also lead to the formation of prostaglandin E_2 (PGE_2) and 5-hydroxytryptamine (5-HT or serotonin), which mediate H_2O and electrolyte transport out of the intestinal cells (Dubreuil, 2008) (**Figure 3C**). The importance of STb and its contribution to diarrhea in 5-days-old pigs was evaluated in an additive model, in which the authors demonstrated that both STb and LT contribute to diarrhea, since only STb^+LT^+ strains caused appreciable diarrhea (Zhang et al., 2006). However, further studies indicated that STb is a more significant contributor to diarrhea in weaned pigs, because a significantly higher intestinal fluid response was observed in LT^+STb^+ ETEC infected piglets as compared to LT^+STb^- infected piglets (Loos et al., 2012; Erume et al., 2013). Intriguingly, increased fluid accumulation by STb seems to reduce the adherence of bacteria to the mucosal surface. In addition, STb impairs the epithelial barrier function through downregulating and/or redistributing the tight junction (TJ) proteins ZO-1, claudin-1, and occludin (Ngendahayo Mukiza and Dubreuil, 2013; Nassour and Dubreuil, 2014). The ability of STb to trigger elevated intracellular Ca^{2+} levels seems to accelerate this process (Nassour and Dubreuil, 2014).

1.4.4 Other virulence factors

In addition to the well-known virulence factors, recent research has identified novel virulence factors, including adhesins, enterotoxins and other potential virulence factors discovered in human ETEC isolates. Indeed, ETEC strains can also produce afimbrial adhesins, such as adhesin involved in diffuse adherence (AIDA-I) and porcine attaching-and-effacing associated (Paa) adhesin. Also other types of enterotoxins were identified in porcine ETEC strains, including enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) and Shiga Toxin type 2e (Stx2e) (Kaper et al., 2004).

AIDA-I was originally identified from a different group of pathogenic *E. coli*, called diarrheagenic *E. coli* (DAEC). AIDA-I is a bacterial auto-transported protein, which mediates diffuse adherence to host receptors present on small intestinal epithelial cells (Benz and Schmidt, 1992). The presence of AIDA-I is usually associated with F18 and/or Stx2e as well as EAST1 (Mainil et al., 2002; Zhao et al., 2009). The adhesin Paa is a bacterial outer membrane protein. A high prevalence of the *paa* gene has been detected in ETEC strains isolated from pigs with diarrhea (Vidotto et al., 2013; Zhang et al., 2007; Boerlin et al., 2005). In addition, the *paa* gene was more associated with F4-positive isolates (64.7%) than with F18-positive isolates (5.9%) (Byun et al., 2013a). Despite this, the virulence significance of *paa* in PWD has not been well characterized.

The EAST1 toxin is commonly found in several groups of diarrheagenic *E. coli* including EAEC, EPEC, ETEC and STEC. Although highly prevalent in ETEC strains (Zhang et al., 2007), the pathogenic significance of EAST1 in diarrheal pigs is still a matter of debate. Recent work implicated the EAST1 toxin does not contribute to the increase of cAMP or cGMP levels in the porcine intestinal epithelial cell line IPEC-J2 or the human intestinal epithelial cell line T-84 (Ruan et al., 2012). Moreover, EAST1 seems not to contribute to diarrhea, since a significantly higher prevalence of this toxin was found in rectal swab samples of healthy piglets as comparison to diarrheic piglets (Zajacova et al., 2012). Stx2e is a member of the Shiga toxin family, a group of ribosome inactivating toxins that cause vascular damage. Although it is often found

(together with LT and/or ST enterotoxins) in ETEC strains isolated from pigs with PWD, Stx2e is thought to primarily cause edema disease in young pigs (Moxley, 2000; Jeffrey, 2012).

In particular, several other potential virulence factors, which were discovered in human ETEC strains, may contribute to the virulence of ETEC in piglets. For instance, the ETEC autotransporter A (EatA) mediates the degradation of MUC2 and other mucins and thus promotes bacterial colonization of the small intestinal epithelium and accelerates toxin access to the epithelial surface (Kumar et al., 2014; Luo et al., 2014). Interestingly, this antigen has more than 80% homology with SepA, which is encoded on virulence plasmids and detected in 92% of porcine O149 ETEC isolates (Boerlin et al., 2005). Moreover, in porcine ETEC strains this plasmid encodes drug resistance and other virulence-related factors, such as STa and Paa (Boerlin et al., 2005). Another protein EtpA, identified in human ETEC strains, functions as a molecular bridge between conserved regions of flagella and host cell receptors and probably assists in attachment of ETEC to the intestinal epithelium (Fleckenstein et al., 2006; Roy et al., 2009). This novel virulence protein probably also participates in the colonization of pigs by ETEC, since this gene has been detected in 25% of porcine ETEC isolates (Lin, 2011).

1.5 Prevention and control of F4⁺ ETEC infection in pigs

In general, strategies for the prevention of F4⁺ ETEC-associated neonatal and postweaning diarrhea should include access to safe water and feed, sanitation measures, antibiotic treatment and vaccination. Unweaned piglets seem to be the key source of pathogenic *E. coli*, because routine cleaning and disinfection of the piggery is insufficient to break the infection cycle (Hampson et al., 1987). Thus, even with a good hygiene management in the piggery, F4⁺ ETEC cannot be completely eliminated. Fortunately, newborn piglets can be protected against ETEC infection by a passive colostral and lactogenic immunity obtained by vaccination of the sow (Rutter and Jones, 1973; Deprez et al., 1986). Indeed, several maternal ETEC vaccines are on the market, either composed of inactivated bacteria or purified fimbriae with or without LT

(Nagy and Fekete, 2005). However, this maternal immunity immediately disappears at the moment of weaning and hence, weaning piglets (3-5 weeks of age) become vulnerable again to F4⁺ ETEC infections. Despite the search for alternative strategies to prevent post weaning F4⁺ ETEC infections, antibiotics are still essential to control and treat *E. coli* infections. However, inappropriate usage of antibiotics can easily lead to antimicrobial resistance (van Duijn et al., 2011). Indeed, antimicrobial resistance to apramycin, neomycin, trimethoprim, trimethoprim-sulfamethoxazole and colistin has been increasingly observed in ETEC strains causing PWD (Zhang et al., 2014). There are also geographical differences in antibiotic resistance in ETEC, which probably correlate to 1) antibiotic usage and 2) the type of antibiotic that is preferentially used in these countries/areas (Boerlin et al., 2005; Do et al., 2006; Okello et al., 2015).

As antimicrobial resistance is a significant threat to human health (Woolhouse et al., 2015), alternative approaches to prevent PWD are being developed, including bacteriophage therapy, prebiotics and probiotics, modification of the receptor attachment sites or breeding of F4R negative pigs. Several studies have found a beneficial role of bacteriophage therapy in weaned pigs as they can significantly reduce the incidence of diarrhea and the bacterial number in feces after ETEC infection (Jamalludeen et al., 2009; Cha et al., 2012). However, until now, very limited phages specific for ETEC have been found (Begum et al., 2010). Moreover, the application of bacteriophage treatment may also be limited due to the strain specificity of these phages, which probably results in a protective effect against one specific ETEC strain. Beneficial effects of probiotics, such as *Lactobacillus*, have also been reported in PWD (Taras et al., 2006; Roselli et al., 2007; Konstantinov et al., 2008; Lessard et al., 2009; Zanello et al., 2011a, b; Lee et al., 2012; Trckova et al., 2014). Although not very clear, this protective effect of probiotics may be attributed to their capacity to reduce F4⁺ ETEC attachment to the epithelia (Roselli et al., 2007; Daudelin et al., 2011), to inhibited ETEC induced tight junction changes (Yu et al., 2015a), and/or to enhance innate immunity by promoting the intestinal expression of cytokines, such as IL-6 (Daudelin et al., 2011; Badia et al., 2012; Zhu et al., 2014; Zhou et al., 2015). Despite these promising results, other studies reported contradictory results, showing that feeding those probiotic strains had no or no consistent protection against PWD

(De Cupere et al., 1992; Bekaert et al., 1996; Walsh et al., 2007). Moreover, the control of storage/fermentation conditions of the probiotics, which are critical to ensure reproducibility, are difficult to achieve with commonly used feed processing technologies (Kenny et al., 2011).

As the expression of the F4 receptors is genetically determined and inherited in a dominant way (Bijlsma and Bouw, 1987; Gibbons et al., 1977), DNA markers were developed to select for F4-resistant pigs (Jørgensen et al., 2003; Rasschaert et al., 2007). Recently, DNA marker in region near the *muc13* gene on pig chromosome 13 has been found to be in almost complete linkage disequilibrium with F4ab/ac ETEC susceptibility (Goetstouwers et al., 2014). However, this marker is not linked to F4ad ETEC susceptibility and thus cannot protect against F4ad ETEC infection. Meanwhile, it's time consuming and expensive to breed F4R⁻ piglets.

In addition to these strategies, oral vaccination with subunit or live avirulent/attenuated F4⁺ ETEC strains has also been applied against PWD. Immunization of weaned pigs with F4 fimbriae induces F4-specific antibodies (Van den Broeck et al., 1999a,b) and protects these piglets against challenge infection (Verdonck et al., 2002, 2004b). This strategy may fail in certain pigs, which do not show an immune response against F4 fimbriae, but are nevertheless vulnerable to F4⁺ ETEC infection (Nguyen et al., 2013). Since F4⁺ ETEC occurs shortly, mostly 3-10 days, after weaning, an ideal vaccine based on these fimbriae should be given to suckling piglets and avoid interference from maternal antibodies (Melkebeek et al., 2013). Recent work in our lab indicated that maternal F4-specific antibodies enhance systemic recall immune responses upon oral immunization of weaner piglets with F4 fimbriae (Nguyen et al., 2015). However, future research should focus on the development of administration strategies to orally immunise suckling piglets with F4 fimbriae and further elucidate if in this context maternal immunity promotes local and systemic ETEC-specific immunity. Since 2008, a live vaccine, consisting of avirulent *E. coli* bacteria that express F4 fimbriae but not the enterotoxins, was shown to be protective against F4⁺ ETEC-induced PWD and subsequently was commercialized (www.prevtectmicrobia.com). However, this vaccine needs to be administered to weaning piglets and as mentioned above, an ideal vaccine should be given during the

suckling period to avoid interference of the maternal immunity.

Compared to live avirulent vaccines, live attenuated *E. coli* strains are more effective to control diarrhea after F4⁺ ETEC infection (Ruan and Zhang, 2013). Similarly, immunization with a fusion protein composed of FaeG and LT elicited corresponding antibodies and significantly reduced colonization of the porcine intestine after challenge with a virulent F4⁺ ETEC strain and the severity of diarrhea (Ruan et al., 2011). These findings suggest that effective ETEC vaccines may need to induce both anti-adhesin immunity to block ETEC adherence and anti-toxin immunity to neutralize enterotoxins. Thus, to develop an efficient vaccine to protect piglets from F4⁺ ETEC infections, more basic research on the immune system against ETEC virulence factors and host ETEC interactions is necessary.

Chapter 2. Immunity against F4⁺ ETEC

2.1 Encounters of the fifth kind: epithelial responses to ETEC

The mucus layer is considered the first line of defense between the luminal bacteria and the host cells, and also serves as the initiation surface for host-microbe interactions. The mucus mainly comprises mucins, which can be further divided into secreted mucins (like MUC2) and membrane-bound mucins (e.g. MUC3). Indeed, the mucus layer serves as a substrate for the growth, adhesion and protection of the trillions of commensal bacteria that are present in the lumen in the intestinal tract. Undeniably, F4R present in the jejunal and ileal mucus also provide an adhesion site for F4⁺ ETEC (Conway et al., 1990). However, compared to the mucus layer in the stomach and colon, the mucus layer in the jejunum and ileum is much thinner and more loosely (Atuma et al., 2001; McGuckin et al., 2011). As this loosely attached mucus is quickly replenished (Atuma et al., 2001; Johansson et al., 2008), F4R on the mucus in the small intestine seems to facilitate the removal of ETEC by effective peristalsis in the intestine. Therefore, by offering binding sites similar to those of IECs, mucus may prevent ETEC adhesion to the underlying epithelial cells.

Intestinal epithelial cells (IECs) are the first cells encountering intestinal pathogens. These cells not only form a physical barrier preventing passage of macromolecules and pathogens to the underlying tissues, they can also sense and respond to microbial stimuli to reinforce their barrier function through secretion of cytokines and chemokines and to participate in the coordination of appropriate immune responses, ranging from tolerance to anti-pathogen immunity (Peterson and Artis et al., 2014). Despite the importance of IECs in pathogen recognition, only a few studies assessed the response of porcine IECs towards ETEC. These studies were largely carried out with cell lines isolated from porcine small intestine, including IPEC-J2, IPEC-1 or IPI-2I cell lines (Berschneider, 1989; Gonzalez-Vallina et al., 1996; Kaeffer et al., 1993). Indeed, most ETEC strains can adhere to the IPEC-J2 cells as well as IPEC-1 cells, although the degree of ETEC adherence to these cells is strain-specific (Koh et al., 2008). ETEC adherence triggered mRNA expression of genes involved in the innate immune response, such as IL-8 and IL-1 α , in IPEC-J2 cells (Geens and Niewold, 2010).

Similarly, our lab demonstrated ETEC infection stimulated the secretion of IL-8 and IL-6 by IPEC-J2 monolayers (Devriendt et al., 2010b). However, IL-1 β secretion was unchanged and TNF- α secretion was not observed in the culture supernatants 24 hours after stimulation with F4⁺ ETEC (Devriendt et al., 2010b). As TNF- α secretion was observed in the IPEC-J2 cell culture supernatants 3 h after incubation with F4⁺ ETEC (Zhang et al., 2015), TNF- α secretion may have occurred earlier. As significant secretion of TNF- α was observed in the IPEC-J2 cell culture supernatants 3 h after incubation with F4⁺ ETEC (Zhang et al., 2015), TNF- α secretion may have occurred earlier. Zhou and colleagues also found that the mRNA expression of IL-6, IL-8 and IL-1 α were increased after infection in IPEC-J2 cells stimulated with F4ab/F4ac, but not F18ac expressing ETEC strains (Zhou et al., 2012). Moreover, transcripts of granulocyte-macrophage colony stimulating factor (GM-CSF) and several chemokines, including chemokine (C-X-C motif) ligand 2 (CXCL2), chemokine (C-C motif) ligand 2 (CCL2) and CCL20, were highly induced by F4⁺ ETEC strains, especially by F4ac (Zhou et al., 2012; Sargeant et al., 2011). On the contrary, the expression of these genes was either undetected or slightly changed by F18ac⁺ ETEC in IPEC-J2 cells. These results indicated that adhesion of ETEC to the IECs is necessary to trigger epithelial responses because F18⁺ ETEC strains are unable to bind IPEC-J2 cells (Koh et al., 2008; Rasschaert et al., 2010).

In addition to IPEC-J2 cells, IPEC-1 cells and IPI-2I cells also respond fast (3h after incubation) to F4⁺ ETEC infection by upregulating mRNA expression of the chemokines CCL2, CCL20, CXCL2, CXCL10, CXCL12 and the cytokines IL-6, IL-1 α , IL-8 and TNF- α (Zanello et al., 2011a,b). Similarly, increased IL-6, IL-1 α , IL-8 and TNF- α transcripts were also found in IPEC-1 as early as 1h after F4⁺ ETEC infection (Taranu et al., 2015). Moreover, IL-17 and IL-10 mRNA expression was also induced, although it is not clear which IL-17 family member these authors investigated (Taranu et al., 2015). However, IL-17A mRNA was found in porcine IECs (Moue et al., 2008) and the human enterocyte cell line Caco-2 (Chow and Lee, 2008; Danesi et al., 2010). In contrast, IPEC-1 cells did not produce IL-17A mRNA upon ETEC infection (Zanello et al., 2011a). However, F4⁺ ETEC (F4ac⁺LT⁺STa⁻STb⁻) failed to enhance IL-8 and

TNF- α mRNA expression in another intestinal porcine cell line IPI-2I in the first 2 hours (Pavlova et al., 2008). In contrast, F4⁺ ETEC (F4ad, LT⁺ and STb⁺) significantly induced the mRNA expression of TNF- α and other inflammatory cytokines (IL-1 α , IL-6) and chemokines (CCL20, CCL2, CXCL2 and CXCL8) 3 hours after incubation with the same cell line (Badia et al., 2012). This disparity in TNF- α mRNA expression may indicate that incubation time and/or enterotoxins influence the epithelial response during ETEC infection. Given that IPEC-J2 cells and IPEC-1 are isolated from the jejunum of a day old piglet (Berschneider, 1989, Gonzalez-Vallina et al., 1996), while IPI-2I cells were isolated from the ileum of an adult pig (Kaeffer et al., 1993), the source of the cell lines should be considered when investigating and comparing epithelial responses to ETEC (Brosnahan and Brown, 2012). Nevertheless, the above findings clearly indicate that intestinal epithelial cells respond to ETEC infection by production of a variety of pro-inflammatory cytokines and chemokines, which may attract and activate innate immune cells, such as neutrophils (IL-8) and CCR6⁺ DCs (CCL20) (Hammond et al., 1995; Iizasa and Matsushima, 2001; Mowat and Agace, 2014).

While the above studies have looked at the epithelial response to ETEC *in vitro* using cell lines, limited studies have been performed concerning the early local immune response in the intestine upon ETEC infection *in vivo*. In order to investigate the early immune responses in the host after infection with ETEC, the *in vivo* small intestinal segment perfusion (SISP) technique was developed (Nabuurs et al., 1993; Niewold et al., 2005). Using this model, the mucosal expression of the pancreatitis associated protein, (PAP) was increased in the intestinal segments as early as 4 hours after F4⁺ETEC (O149:K91, F4ac⁺LT⁺STb⁺) perfusion (Niewold et al., 2005; Loos et al., 2012). This protein is normally upregulated after inflammation (van Beelen Granlund et al., 2013) and functions as an antimicrobial protein (Cash et al., 2006; Brandl et al., 2008; Vaishnava et al., 2011). Besides PAP, matrix metalloproteinase-1 (MMP-1) and IL-8 are also induced after ETEC perfusion in the SISP technique (Loos et al., 2012) and probably contribute to the modulation of the chemokine and cytokine gradients that drive inflammatory cell recruitment (Elkington et al., 2005). In addition, IL-17A and IL-1 β mRNA were also upregulated 4 hours in the intestinal segments after perfusing

with F4⁺ETEC (Loos et al., 2012). Moreover, the early IL-17A and IL-1 β response seems to be associated with STb, because only the wild type and STb⁺ ETEC mutant strains elicited this mRNA expression profile (Loos et al., 2012). Recently, F4⁺ ETEC was found to induce mRNA expression of antimicrobial peptides, like β -defensin 2, α -defensin-5 (HD-5) and PR-39, in different tissues of infected pigs (liver, ileum, spleen and thymus) or humans (duodenal epithelium) (Shirin et al., 2011; Gao et al., 2014). However, the intestinal mRNA expression profile was assessed on RNA obtained from total tissues in these studies and thus no information on cell-specific responses were obtained.

2.2 Innate immunity

In response to recognition of bacterial ligands by intestinal epithelial cells and resident sub-epithelial immune cells, such as mast cells, macrophages and dendritic cells, innate immune responses are initiated, resulting in a rapid burst of pro-inflammatory cytokines and chemokines (e.g. IL-6, IL-8, and TNF- α) into the surrounding tissue and circulation (Medzhitov and Janeway, 1997). These released pro-inflammatory mediators recruit effector cells such as neutrophils to the site of infection where, via multiple mechanisms, they aid in containing and eventually clearing the pathogen. As mentioned above porcine intestinal epithelial cells secrete these pro-inflammatory mediators in response to ETEC adhesion, presumably attracting innate immune cells to the site of infection. Indeed, upon ETEC infection, neutrophils are attracted to the lumen of the gut (Rose and Moon, 1985), the intestinal wall and the mesenteric lymph nodes (Elsinghorst and Kopecko, 1992; Konstantinova et al., 2008; McLamb et al., 2013). Neutrophils can decrease the viability of ETEC and this effect seems to depend on the serotype of ETEC, but also correlates with the presence of some plasma factors (Ondrackova et al., 2012). In addition, in the lamina propria, mast cell numbers are also increased and are activated upon ETEC challenge (McLamb et al., 2013). Interestingly, this early influx of neutrophils and mast cells and cytokine production are influenced by the weaning age of the piglets during ETEC challenge infection (McLamb et al., 2013).

Another important innate immune cell is the DC, which bridge innate and adaptive immunity as these cells have the unique ability to activate naïve T cells. Dendritic cells patrol the peripheral tissues and upon encountering pathogens, they endocytose ligands and migrate from the periphery to the mesenteric lymph nodes in a CCR7-dependent manner. During this migration, the DCs undergo a maturation process, which is characterized by an upregulation of MHCII and several costimulatory molecules, required to properly activate T cells, and a change in their cytokine/chemokine secretion profile. Indeed, depending on the encountered pathogen, DCs secrete a defined set of cytokines, which drive the differentiation of naïve T cells into an effector phenotype most suitable to eradicate the pathogen. In the gut, these DCs further have the ability to endow T cells with gut homing properties, a mechanism which requires retinoic acid. A few studies have addressed the effect of F4⁺ ETEC or its virulence factors on DC maturation, primarily because it was demonstrated that F4 fimbriae are taken up by gut SIRPα⁺ cells, which include DCs (Snoeck et al., 2008). Until now, no studies have investigated the effect of ETEC infection in pigs on DC function *in vivo*. However, *in vitro* stimulation of monocyte-derived dendritic cells (MoDCs) with live F4⁺ ETEC elicited mRNA expression of several cytokines (TNF-α, GM-CSF, IL-6, IL-10), chemokines (CCL17), chemokine and pattern recognition receptors (CCR7, TLR2, TLR4), the B-cell regulatory factors a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF) (Badia et al., 2012). This indicates that F4⁺ ETEC triggers DC maturation. In addition, purified ETEC-derived F4 fimbriae and flagellin trigger maturation of MoDCs as well as small intestinal lamina propria CD11R1⁺ APCs, as evidenced by an upregulated activation marker expression (MHCII, CD40, and CD80/86) and an enhanced ability to induce T cell proliferation (Devriendt et al., 2009, 2010a). On top of neutrophils and dendritic cells a few studies suggest that NK cells as well as γδ T cells may participate in the intestinal immune response to F4⁺ *E. coli* infection (Vijtiuk et al., 2002).

2.3 Adaptive immunity

Innate immunity is in general, however, insufficient to protect the host against an invading pathogen, has a broad specificity and does not induce long-term memory. In

contrast, adaptive immunity triggers antigen-specific immune responses and has the potential to establish long-term memory responses. Adaptive immune cells consist of T and B cells, which can mature into antigen-specific immune cells. As mentioned above, most microbial components are endocytosed by antigen-presenting cells (APCs), processed and presented to T cells. While foreign molecules originating from intracellular pathogens are presented to CD8⁺ cytotoxic T cells via MHC I, antigens derived from extracellular pathogens are presented via MHC II to CD4⁺ T helper (Th) cells, resulting in their differentiation into effector cells. The differentiation of naïve CD4⁺ Th cells into effector Th cells is initiated by the engagement of their T cell receptor (TCR) by peptide-MHC II complexes and costimulatory molecules in the presence of specific cytokines produced by the innate immune system upon encounter of particular pathogens (Korn et al., 2009). Currently, at least four types of CD4⁺ Th cells exist: Th1, Th2, Th17 and regulatory T cells (Tregs). These effector T cells are characterized by subset-specific transcription factors, which regulate their differentiation, and a distinct cytokine secretion profile (Zhu et al., 2010) (**Figure 6**).

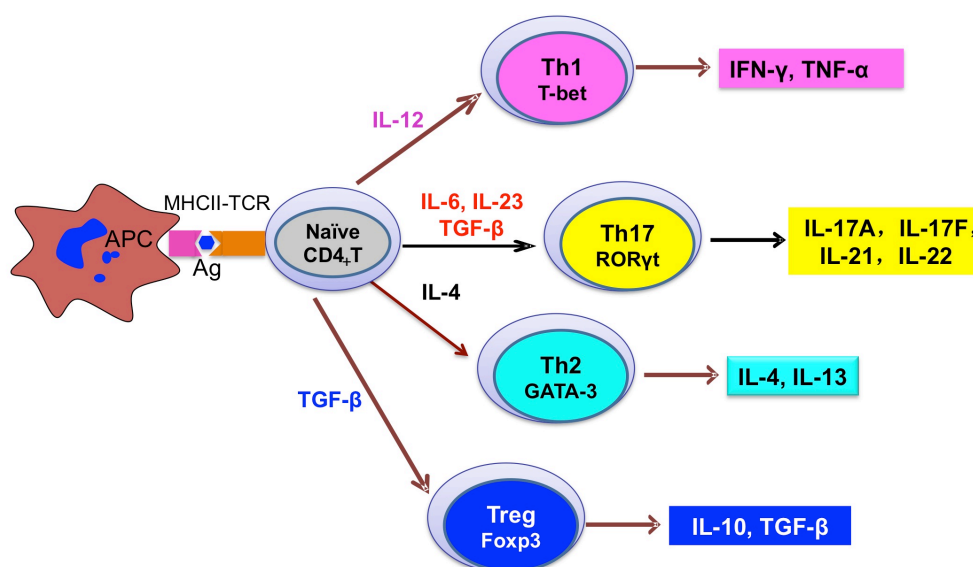


Figure 6. CD4⁺ Th cells differentiation. Antigens are first captured and presented by MHCII on the surface of APCs. This exposed antigen can be recognized by the TCR on the surface of the naïve CD4 T cells. Then, these CD4⁺ T cells are activated, and differentiate into several lineages of T helper (Th) cells driven by the activation of distinct transcription factors by certain cytokines directed by. For example, IL-12 and T-bet play an important role in the induction of Th1 cells, while IL-4 and GATA-3 are critical for the development of Th2 cells. The induction of Tregs from naïve CD4⁺ T cells relies on their activation in the presence of TGF-β and the transcription factor Foxp3. The differentiation of Th17 cells is triggered by the transcription factor RORγt in the presence of TGF-β, IL-6 and IL-23. Upon differentiation and activation, each Th subset produces lineage-specific cytokines.

2.3.1 T and B cell responses

As cytokines and transcription may reveal the type of immune response initiated by the host to counter the pathogenic insult, several studies examined the cytokine responses as well as the T cell responses in the mucosal sites after F4⁺ETEC infection. An F4⁺ ETEC infection is usually self-limiting and resolves within 7-8 days, so the induction of an ETEC-specific immune response should have occurred earlier. Indeed, intestinal cytokine responses (IFN- γ , TNF- α , IL-6, IL-4, TGF- β) were mainly observed at day 4, but not at day 1 and 7 after ETEC infection (Daudelin et al., 2011; Gao et al., 2013; Zhu et al., 2014; Zhou et al., 2015). The enhanced expression of IL-6, TNF- α and IFN- γ after F4⁺ ETEC infection was also observed in serum 48 hours after infection (Lee et al., 2012). These results might suggest the importance of time on the investigation of cytokine responses after ETEC infection.

In addition to the evaluation of cytokine responses upon ETEC infection, a few studies investigated changes in the composition of the T cell population in blood and intestinal tissues upon ETEC infection. Although in peripheral blood no changes were observed 6 days post infection, a significant increase of CD4⁺CD8⁺ T cells was found in the jejunal peyer's patches (JPP) one week post infection (Zhu et al., 2014), indicating the induction of antigen-specific memory T cell responses. The composition of these memory T cells is not clear, but Foxp3⁺ Tregs are probably not required for the protection of the host at day 7 after F4⁺ ETEC infection (Zhou et al., 2015). Interestingly, an increased production of IL-17A mRNA, but not of IL-4, IL-10, T-bet, and IFN- γ , was detected in the intestine 7 days after F4⁺ETEC infection (Zhou et al., 2015), implying that IL-17A producing Th17 cells may be involved in protection against ETEC infection.

Generally, activated Th cells co-operate with B cells for the production of antibodies, which opsonize extracellular microbes and neutralize their toxins. Many *in vivo* models have shown that pathogen-specific secretory IgA (SIgA) is induced to clear bacterial pathogens and that these SIgA responses are required to protect the host against a subsequent infection at the mucosal surfaces. Indeed, rapid F4 fimbriae-specific IgA and IgG antibodies are induced in the intestinal tissues and serum after F4⁺ ETEC infection in pigs, and these F4 fimbriae-specific SIgA responses are

sufficient to protect the piglets against infection (Van den Broeck et al., 1999a; Verdonck et al., 2002). Intriguingly, the induction of these fast F4 fimbriae-specific IgA responses after F4⁺ ETEC infection can be replicated by oral immunization with F4 fimbriae (Van den Broeck et al., 1999a,b). Moreover, oral immunization with F4 can also induce F4 specific IgG and IgM production in the serum and in the small intestine (Van den Broeck et al., 1999a,b; Delisle et al., 2012). Since the antibody responses to protein antigens require the help from antigen-specific Th cells, a few studies assayed Th cell differentiation after ETEC infection or stimulation with ETEC virulence factors. Upon stimulation of naive PBMCs with F4 fimbriae, the Th1/Th2 cytokine response was investigated and only IFN- γ mRNA levels were upregulated in the first 72 h, implying a Th1 directed response (Verfaillie et al., 2001). However, as the F4 fimbriae preparation could have been contaminated with LPS, the IFN- γ mRNA induction may have been caused by LPS instead of F4 fimbriae (Kato et al., 2004; Choi et al., 2002). In humans, increased levels of TNF- α , IL-6 and IFN- γ are associated with a longer duration of ETEC infection, while increased IL-8 expression may be essential to resolve ETEC infections (Long et al., 2010). Due to the limited studies, it remains unclear which Th response (Th1, Th2, Th17 or Tregs) contributes to the induction of protective F4-specific SIgA responses. However, Th17 cells seem promising candidate as accumulating data implicate these cells in the induction of SIgA and subsequent protection against pathogens at the mucosal sites (Jaffar et al., 2009; Cao et al., 2012; Hirota et al., 2013; Cao et al., 2015). Thus, in the next section, the current knowledge on Th17 cells and their potential role in triggering mucosal immunity against bacterial infection will be summarized.

2.4 Role of Th17 cells in mucosal immunity

2.4.1 Differentiation of Th17 cells

In humans and mice, differentiation of Th17 cells from naive T cells is driven by transforming growth factor- β (TGF- β) and IL-6 (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006). These two cytokines activate STAT-3 and induce the expression of the transcription factor ROR γ t, which triggers transcription of the IL-17 gene in naïve T cells (Mathur et al., 2007). However, in the absence of IL-6, IL-21 in

combination with TGF- β can function as an alternative signal for the induction of Th17 cells (Korn et al., 2007). Because IL-21 is mainly produced by Th17 cells, they can enhance their own differentiation through an autocrine amplification loop. In addition, these Th17 cells can be expanded and sustained in the presence of IL-23 (Stritesky et al., 2008).

2.4.2 Th17 cells and mucosal immunity

Although Th17 cells and their cytokines were first identified in many different autoimmune and auto-inflammatory diseases, the discovery of patients with a primary immunodeficiency in the IL-17 pathway revealed the critical role of Th17 cells in defense against certain fungal and bacterial infections (reviewed in McDonald, 2012; Maródi et al., 2013). Now, there is growing evidence that Th17 cells are critical for host defense against bacterial, fungal and viral infections, and particularly at mucosal surfaces (**Table 6**). Th17 cells induce the recruitment of neutrophils and barrier enhancement of IECs, leading to enhanced clearance of extracellular pathogens in concert with other immune cells, such as IgA-secreting plasma cells. Th17 cells preferentially secrete IL-17A, IL-17F, IL-21 and IL-22 (McGeachy and Cua, 2008). IL-21 contributes to IL-17A production (Wei et al., 2007), IgA production as well as B-cell trafficking to the intestine (Cao et al., 2015). IL-22, on the other hand, alone or in combination with IL-17A enhances the expression of antimicrobial peptides, including β -defensin 2 (BD-2), S100A7, S100A8, and S100A9, by human keratinocytes (Liang et al., 2006). IL-17A and IL-17F belong to the IL-17 cytokine family, which consists of six members (detailed below) (Jin and Dong, 2013; Iwakura et al., 2011). IL-17A is the best characterized IL-17 cytokine family member and was found to enhance the epithelial barrier by promoting the expression of tight junction proteins, antimicrobial peptides (AMPs) and pro-inflammatory cytokines (Cua and Tato, 2010; Lee et al., 2015). In addition, Th17 cell/ IL-17A is also involved in pIgR expression and secretory IgA levels, thus protecting the host against pathogens (Jaffar et al., 2009; Cao et al., 2012). As shown in **Table 6**, a protective role of IL-17F was also found against several mucosal pathogens, such as *Klebsiella pneumonia* and *Mycoplasma pneumonia* in

the airway epithelium and *Citrobacter rodentium* in the colon (Aujla et al., 2008, Wu et al., 2007; Ishigame et al., 2009).

Th17 cells, however, are not only induced after pathogen infection but also driven by commensal microbiota. In the gut, at least in humans and mice, segmented filamentous bacteria (SFB), are essential to the development of constitutive Th17 cells (Ivanov et al., 2008, 2009; Gaboriau-Routhiau, 2010; Atarashi et al., 2015). The adhesion property of SFB to absorptive epithelial cells in the ileum and cells overlying PP was demonstrated (Jepson 1993; Chase and Erlandsen, 1976) and was recently demonstrated to correlate with the induction of intestinal Th17 cells (Ivanov et al., 2009; Atarashi et al., 2015; Sano et al., 2015). In addition, *C. rodentium* and EHEC O157 also promote the induction of intestinal Th17 cells through adhesion to IECs (Atarashi et al., 2015). Moreover, serum amyloid A (SAA) derived from IECs is required to enhance the differentiation and effector function of Th17 cells (Atarashi et al., 2015; Sano et al., 2015). It is unclear whether resident Th17 cells exhibit specificity for microbial antigens. However, colonization with SFB and Th17 induction are required for the host survival after *C. rodentium* infection, probably by reducing the capacity of *C. rodentium* to grow and/or invade in colonic tissue (Kolls et al., 2008; Zheng et al., 2009; Ivanov et al., 2009). Moreover, these homeostasis Th17 cells may also exert their role in mucosal immunity as their hallmark cytokine IL-17A was highlighted in promoting cytokine production and protection against extracellular pathogens and in colitis by fortifying the epithelial barrier (Lee et al., 2015; Maxwell et al., 2015). Th17 related inflammation is a beneficial host response to infection, but the commensal gut bacteria induced Th17 cells might contribute to inflammatory disease and tissue injury in the absence of a proper control. Recent work demonstrated that Th17 cells can transdifferentiate into Tregs to prevent overinflammation (Gagliani et al., 2015), implying the commensal derived Th17 cells may control the intestinal inflammation through this mechanism. As ETEC can also adhere to the IECs, It would be interesting to evaluate the Th17 and IL-17 response after ETEC infection in pigs.

Table 6 Th17 cells/ cytokines in host defense

Host defence against	Main effector cytokines	Evidence/mechanisms	References
<i>Klebsiella pneumoniae</i> (KP)	IL-23p19	IL-23p19 KO and IL-17R KO are greatly susceptible to KP	Happel et al., 2005
	IL-17A/IL-22	IL-17A KO are greatly susceptible to KP, and IL-17A promotes neutrophil recruitment in infection with KP	Aujla et al., 2008
	IL-17A	Overexpression of IL-17A resulted in local induction of cytokines, chemokines, recruitment of polymorphonuclear leukocyte, and enhanced bacterial clearance and survival after KP	Ye et al., 2001b
	IL-17R signaling	Induction of chemokines and neutrophil recruitment	Ye et al., 2001a
<i>Citrobacter rodentium</i>	Th17 cells	<i>Citrobacter rodentium</i> can induce Th17 cells in the absence of IL-23p19 in the gut mucosa, but no protective immunity	Mangan et al., 2006
	IL-22	Induction of chemokines and antimicrobials	Zheng et al., 2008 ; Ishigame et al., 2009
<i>Mycoplasma pneumoniae</i> (Mp)	IL-17A/IL-17F	IL-17 production is essential in neutrophil recruitment and against respiratory Mp infection	Wu et al., 2007
		IL-17/IL-17F production is IL-23-dependent in an acute Mp infection	
<i>Candida albicans</i>	IL-17	Induction of chemokines and anti-microbials and neutrophil recruitment	Conti et al., 2009
	Natural Th17 cells and $\gamma\delta$ T cells produce IL-17	Rapid IL-17A induction	Conti et al., 2014
	Th17 cells and IL-17A	Both CD4 ⁺ Th17 and other IL-17A producing cells protect from <i>Oropharyngeal candidiasis</i> infection	Hernández-Santos et al., 2013
	IL-23-IL-17A axis	Chemokine induction and recruitment of effector CD4 ⁺ T cells	Rudner et al., 2007
<i>Mycobacterium tuberculosis</i> (Mtb)	IL-17A	IL-17-driven chemokine expression is required to attract protective IFN- γ -producing Th1 cells in Mtb infection	Khader et al., 2007
<i>Mycobacterium bovis</i> bacillus Calmette-Guérin (BCG).	Innate IL-17A- $\gamma\delta$ T cells	Neutrophil-mediated inflammatory response, but also in T cell-mediated IFN- γ production and granuloma formation in response to pulmonary infection by BCG	Umemura et al., 2007
BCG/Mtb	IL-17A- $\gamma\delta$ T cells	Critical role in the prevention of Mtb infection through the induction of mature granuloma formation	Okamoto Yoshida et al., 2010

2.4.3 The IL-17 cytokine family

As mentioned before, in addition to IL-17A and IL-17F, IL-17B, C, D and E (also known as IL-25) also belong to the IL-17 cytokine family. Among the IL-17 family members, IL-17F shares the highest degree of homology with IL-17A (40-55%), followed by IL-17B (29%), IL-17D (25%), IL-17C (23%) and IL-17E (17%) (Kolls and Lindén, 2004). IL-17A and IL-17F are both secreted as disulfide-linked homodimers or heterodimers (**Figure 7**), can bind to the same IL-17 receptor (IL-17R), a heterodimer of IL-17RA and IL-17RC subunits and then mediate pro-inflammatory responses or adaptive immune response, particularly against bacteria and fungi (Iwakura et al., 2011). Compared to IL-17A and IL-17F, the function of IL-17B, IL-17C and IL-17D is less understood. Although Th17 effector cells are best known for their ability to produce IL-17A, other lymphocytes can also produce IL-17A in the presence of common transcription factors ROR γ , including CD8 $^{+}$ $\alpha\beta$ T cells, $\gamma\delta$ T cells (Jensen et al., 2008), LT α i-like innate lymphoid cells (ILCs) (Crellin et al., 2010), natural killer cells (NK) (Passos et al., 2010), and CD3 $^{+}$ invariant natural killer T (iNKT) cells in mice and/or humans (Michel et al., 2007). Moreover, neutrophils (Ferretti et al., 2003), macrophages (Song et al., 2008) and intestinal Paneth cells (Takahashi et al., 2008) were also identified as a cellular source of IL-17A. It is increasingly accepted that this innate IL-17A producing cells are strategically positioned in the barrier tissues, such as lungs, intestines, skin and peripheral lymph nodes, to allow a rapid reaction to pathogens, but also activate and amplify the adaptive immune responses (Lee et al., 2015; Sano et al., 2015). As described in **Table 6**, these innate IL-17A, also acts to promote recruitment and activation of neutrophils that prevent bacterial spread.

Similar to IL-17A, IL-17F is also produced by many cell types, such as memory CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, $\gamma\delta$ T cells, NKT cells, B cells and lymphoid tissue-inducer (LT α i) cells (reviewed in Ota et al., 2014). Besides, colon and bronchial epithelial cells were also identified as a source of IL-17F mRNA and IL-17F protein, respectively (Ishigame et al., 2009; Fujita et al., 2012). Thus, in addition to its function in allergic airway inflammation (Kawaguchi et al., 2001), IL-17F may have a more diverse biological function in the gut. Similarly like IL-17A, IL-17F is also involved in the

development of inflammation and host defense against infection by inducing the expression of genes encoding proinflammatory cytokines (TNF, IL-1, IL-6, G-CSF, and GM-CSF), chemokines (CXCL1, CXCL5, IL-8, CCL2, and CCL7), antimicrobial peptides (defensins and S100 proteins), and matrix metalloproteinases (MMP1, MMP3, and MMP13) from fibroblasts, endothelial cells, and epithelial cells (Iwakura et al., 2011). However, IL-17F occurred earlier than IL-17A during Th17 cell development (Lee et al., 2009c). Not much is known about the role of IL-17A/IL-17F heterodimer. However, their expression as well as IL-17F and IL-17A homodimers is confirmed in the activated human CD4⁺ T cells and human embryonic kidney 293 cells, can induce chemokine GRO- α production in a human bronchial epithelial cell line and IL-6 and CXCL1 expression in mouse embryonic fibroblasts (Wright et al., 2007; Chang and Dong, 2007). Thus, IL-17A/IL-17F heterodimer may displays similar function as IL-17A and IL-17F in chemokine induction and neutrophils recruitment.

IL-17B also appears to participate in the inflammatory responses, since this cytokine can induce the secretion of several pro-inflammatory cytokines, such as TNF- α and IL-1 β , by monocytic THP-1 cells (Li et al., 2000; Shi et al., 2000). It is expressed in the pancreas, small intestine, stomach, and in the spinal cord as well (Li et al., 2000; Moore et al., 2002). As shown in **Figure 7**, IL-17B and IL-17E (IL-25) share a common receptor, IL-17RB (Shi et al., 2000; Huang et al., 2014). This probably explains why IL-17B is able to antagonize the proinflammatory function of IL-25 and prevent overinflammation during acute colitis and *Citrobacter rodentium* infection (Reynolds et al., 2015). Recent work also described the essential role of IL-17B in the promotion of tumorigenesis (Wu et al., 2015; Huang et al., 2014), probably because L-17B–IL-17RB signaling can induce the production of chemokines, which may contribute to the recruitment of macrophages and enhance cancer cell survival. Given that IL-17RB is expressed by mucosal epithelial cells (Shi et al., 2000; Lee et al., 2001; Zhao et al., 2010), IL-17B may also regulate the local immune response in the gut.

IL-17C binds to IL-17RE (**Figure 7**) and activates NF- κ B (Gaffen, 2009; Starnes et al., 2002). It is found in CD4⁺ T cells, DCs and macrophages at inflammatory sites (Hwang and Kim, 2005; Li et al., 2000; Yamaguchi et al., 2007), and seems to mediate

inflammatory responses by these cells. Recently, IL-17C produced by goblet cells and enteroendocrine cells has also been proposed to mediate the intestinal inflammation in IBD patients (Friedrich et al., 2015; Reynolds et al., 2012). In addition, IL-17C can specifically induced in the epithelial cells derived from the trachea, colon, skin and kidney but not in dermal fibroblasts and PBMCs after bacteria or fungal stimulation (Ramirez-Carrozzi et al., 2011; Huang et al., 2015). Moreover, the mRNA expression of their receptor, IL-17RE, also much higher in mucosal organs, including the trachea, lungs and colon but very low in human fibroblasts and PBMCs (Li et al., 2006; Ramirez-Carrozzi et al., 2011). These findings suggest the role of IL-17C in innate immune response in the epithelium by triggering production of chemokines, inflammatory mediators and antimicrobial peptides after bacterial stimulation (*E.coli* and *C. rodentium*) or fungal infection (Ramirez-Carrozzi et al., 2011; Song et al., 2011; Huang et al., 2015). Since, IL-17C and its receptor IL-17RE are specifically induced in the epithelial cells and mucosa (Ramirez-Carrozzi et al., 2011; Huang et al., 2015; Li et al., 2006), it would be interesting to investigate their role in the mucosal immunity during other enteropathogenic infections, such as ETEC.

Similar to IL-17B, IL-17D mRNA is highly detected in several tissues, such as skeletal muscle, brain, adipose tissue, heart, lung, and pancreas and less expressed in bone marrow, kidney, leukocytes, liver, lymph node, spleen, thymus, resting CD4⁺ T cells and resting CD19⁺ B cells (Starnes et al., 2002). Even in activated CD4⁺ T cells and CD19⁺ B cells, IL-17D protein is still poorly expressed. IL-17D can induce the expression of IL-6, IL-8, and GM-CSF in endothelial cells (Starnes et al., 2002). Recently, it was found to functions to recruit NK cells and to mediate tumor rejection (O'Sullivan et al., 2014). The receptor for IL-17D has not yet been identified.

The heterodimer consisting of IL-17RA and IL-17RB serves as the receptor for IL-17E (Rickel et al., 2008) (**Figure 7**). IL-17E is produced by Th2 cells, CD4⁺ and CD8⁺ T cells isolated from caecal patches, mast cells, and eosinophils (reviewed in Reynolds et al., 2010). Compared to the other IL-17 cytokine family members, which can induce the expression of proinflammatory cytokines such as TNF- α and IL-1 β and promote neutrophil migration, IL-17E is mainly involved in promoting Th2 cell

responses and IgE production (Iwakura et al., 2011). Thus, IL-17E induces airway inflammation probably through the induction of eosinophilia but not neutrophils aggregation (Angkasekwinai et al., 2010). Th2 cell cytokine production promoted by IL-17E is important for the protection against nematodes such as *Nippostrongylus brasiliensis* and *Trichuris muris* (Fallon et al., 2006; Zhao et al., 2010). IL-17E produced by intestinal epithelial cells in response to commensal bacteria seems to limit intestinal Th17 cell expansion and IL-22 expressing innate lymphoid cells (ILC) 3 (Zaph et al., 2008; Sawa et al., 2011). Thus, IL-17E may be involved in Th17 cell-mediated host defense mechanisms against bacterial infection. Further investigations are needed to figure out the relationship between this cytokine and Th17 cells.

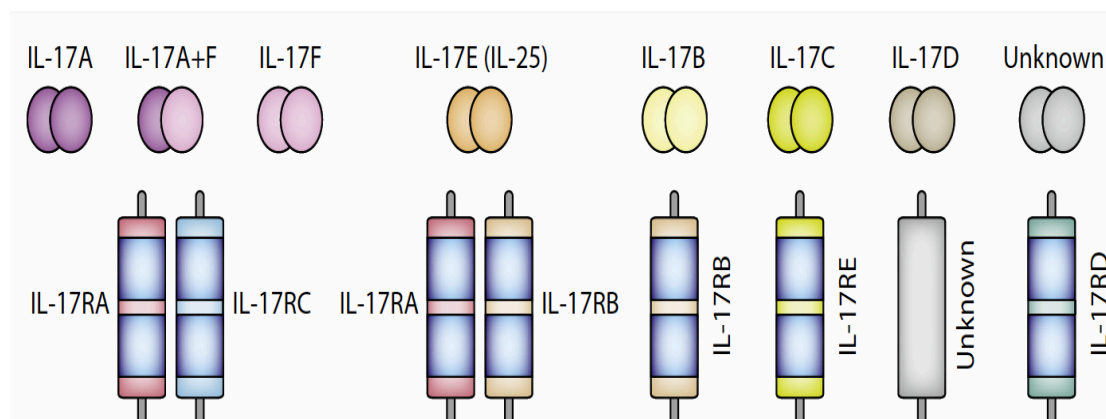


Figure 7. IL-17 cytokines and IL-17 receptor family ligand-receptors

The IL-17 cytokines family has six members from IL-17A to IL-17F and currently five IL-17 receptors (IL-17R) have been identified, from IL-17RA to IL-17RE. IL-17A and IL-17F both are covalent homodimers, but can also form an IL-17A/IL-17F heterodimers. IL-17A, IL-17F and the heterodimer all bind to the same IL-17 receptor, composed of IL-17RA and IL-17RC subunits. IL-25 (IL-17E) binds a receptor complex composed of IL-17RB and IL-17RA, while IL-17RB also binds to IL-17B. IL-17RE specifically binds to IL-17C, while IL-17RD has no known ligand yet. The receptor(s) for IL-17D has not yet been identified. Adapted from Iwakura et al., 2011. Reuse with permission from Elsevier Publishers Ltd: License Number: 3765390424249

2.4.4 Th17/IL-17 response in pigs

In contrast to human and mice, research on porcine Th17 cells and the IL-17 cytokine family is only in its infancy. In 2004, porcine IL-17A was cloned and found to be preferentially expressed in the intestine of the pig (Katoh et al., 2004). Porcine IL-17A strongly induced the mRNA expression of IL-1 β , IL-8, TNF- α , G-CSF, and monocyte chemotactic protein-1 (MCP-1) in splenocytes and mesenteric lymph node (MLN) cells (Katoh et al., 2004). In peripheral blood, porcine CD4⁺ T cells and $\gamma\delta$ ⁺ T cells, but not myeloid cells (CD172a⁺) and B lymphocytes (CD79a⁺), produce IL-17A after stimulation with PMA and ionomycin (Stepanova et al., 2012). Moreover, stimulation of PBMCs with anti-CD3 mAbs in the presence of IL-2 induced IL-17A producing CD4⁺CD25^{dim} T cells, suggesting that the IL-17A producing T cells are mainly composed of Th17 cells and to a lower extent of Tregs (Käser et al., 2012). As in humans, porcine Th17 cells arise from naive CD4⁺ lymphocytes via IL-6 in the context of TGF- β and secrete IL-17A and IL-21 (Kiros et al., 2011).

There are few studies trying to find a role of IL-17A in different diseases in pig. IL-17A seems to be associated with dextran sodium sulfate (DSS)-induced colitis, since it is more expressed in the MLNs of animals with DSS-induced intestinal inflammation and decreased after treatment (Young et al., 2012; Lee et al., 2009a,b; Kim et al., 2010a; Ibuki et al., 2014). However, this inflammatory function of IL-17A seems untenable during bacterial infection, since the IL-17A mRNA expression was either significantly decreased or unaltered in the ileum at day 2 and day 5 after *Salmonella typhimurium* infection (Collado-Romero et al., 2012; Volf et al., 2012). Moreover, a correlation between IL-17A and serum IgA was observed in early-weaned piglets, indicating the potential role of IL-17A in regulating mucosal and systemic IgA production (Levast et al., 2010). Little is known regarding the Th17/IL-17A response during porcine ETEC infections. The heat labile toxin (LT) from a human ETEC strain was found to enhance IL-17A production by human PBMCs in response to antigen or mitogen stimulation (Leach et al., 2012). Similarly, the IL-17A promoting effect of LT was also reported in mice upon *Helicobacter pylori* infection (Sjökvist Ottsjö et al., 2013). Moreover, higher levels of IL-17A are associated with the reduction in bacterial

loads during *Helicobacter pylori* Infection in mice (Sjökvist Ottsjö et al., 2015). In pigs, IL-17A mRNA production in the small intestine was upregulated early during F4⁺ ETEC infection and seems to be dependent on the presence of the enterotoxin STb (Loos et al., 2012). Moreover, elevated IL-17A transcripts are also observed in the intestinal tissues at later stage of F4⁺ ETEC infection (Zhou et al., 2015). However, whether Th17 cells or IL-17A participate in the immune response against an ETEC infection in pigs or other species is still unclear. In addition, the function of the other IL-17 cytokines in pigs is completely unknown.

Aims

Both F4⁺ETEC infection and oral immunization with F4 fimbriae in pigs result in intestinal F4-specific SIgA responses, which protect piglets against a subsequent ETEC infection. At the moment it is unclear how this SIgA response is regulated. Rodent models have recently demonstrated that Th17 cells and their cytokines IL-17A and IL-21 are implicated in the induction of mucosal SIgA responses directed against gut-dwelling pathogens. IL-17A is preferentially expressed in the intestine of young pigs and seems to be associated with IgA production in piglets (Katoh et al., 2004; Levast et al., 2010). Taken together, these data hint that IL-17A produced by Th17 cells may be involved in the intestinal immune responses against ETEC infection.

Previous work at our laboratory indicated that IL-17A may also play an important role during the early phases of ETEC infection. Indeed, transcriptome analysis upon perfusion of the small intestine with F4⁺ ETEC in the small intestinal segment perfusion (SISP) technique clearly showed the upregulation of IL-17A mRNA in the first 4 hours post F4⁺ ETEC infection. Given this early timepoint we speculated that this IL-17A might be produced by intestinal innate immune cells, such as $\gamma\delta^+$ T cells or innate lymphoid cells (ILC) 3. The aim of the present doctoral dissertation was to determine if IL-17A or other IL-17 cytokine family members participated in the immune response during early and late ETEC infection.

The following questions were addressed:

- 1) Which cells produce IL-17A early during F4⁺ ETEC infection? Which virulence factors trigger this innate IL-17A response?
- 2) Does IL-17A play a role in the induction of adaptive immunity during F4⁺ ETEC infection? Given the potent oral immunogenicity of F4 fimbriae, are the latter able to trigger IL-17A responses upon oral administration?

Chapter 4

F4⁺ ETEC infection and oral immunization with F4 fimbriae elicit an IL-17-dominated immune response

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Authors' contributions

YL, BD and EC conceived and designed the experiments. YL carried out the majority of the experiments, was responsible for data collection and drafted the manuscript. YL, VUN and PYFR performed the infection experiment and YL and VUN the immunization experiment. YL, BD and EC were responsible for data analysis and interpretation. BD and EC critically revised the manuscript.

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of post-weaning diarrhea (PWD) in piglets. Porcine-specific ETEC strains possess different fimbrial subtypes of which F4 fimbriae are the most frequently associated with ETEC-induced diarrhea in piglets. These F4 fimbriae are potent oral immunogens that induce protective F4-specific IgA antibody secreting cells at intestinal tissues. Recently, T-helper 17 (Th17) cells have been implicated in the protection of the host against extracellular pathogens. However, it remains unknown if Th17 effector responses are needed to clear ETEC infections. In the present study, we aimed to elucidate if ETEC elicits a Th17 response in piglets and if F4 fimbriae trigger a similar response. F4⁺ ETEC infection upregulated IL-17A, IL-17F, IL-21 and IL-23p19, but not IL-12 and IFN- γ mRNA expression in the systemic and mucosal immune system. Similarly, oral immunization with F4 fimbriae triggered a Th17 signature evidenced by an upregulated mRNA expression of IL-17F, ROR γ t, IL-23p19 and IL-21 in the peripheral blood mononuclear cells (PBMCs). Intriguingly, IL-17A mRNA levels were unaltered. To further evaluate this difference between systemic and mucosal immune responses, we assayed the cytokine mRNA profile of F4 fimbriae stimulated PBMCs. F4 fimbriae induced IL-17A, IL-17F, IL-22 and IL-23p19, but downregulated IL-17B mRNA expression. Altogether, these data indicate a Th17 dominated response upon oral immunization with F4 fimbriae and F4⁺ ETEC infection. Our work also highlights that IL-17B and IL-17F participate in the immune response to protect the host against F4⁺ ETEC infection and could aid in the design of future ETEC vaccines.

4.1 Introduction

In neonatal and recently weaned pigs, ETEC-associated diarrhea is a major cause of illness and mortality and leads to great economic losses in the swine production industry worldwide (Francis, 2002; Nagy et al., 1999; Vu-Khac et al., 2007; Chen et al., 2004). ETEC express fimbriae, which are long proteinaceous appendages radiating from the surface of the bacterium. These fimbriae mediate adhesion to host intestinal epithelia through an interaction with specific receptors present on the brush borders of the small intestinal enterocytes, enabling bacterial colonization (Van den Broeck et al., 1999b). Porcine-specific ETEC strains possess five different fimbrial subtypes, of which F4 fimbriae are the most frequently associated with ETEC-induced diarrhea in piglets (Wang et al., 2006; Fairbrother et al., 2005; Frydendahl et al., 2002). Recent data indicate F4 fimbriae are not merely involved in adherence, but also play a role in the modulation of the immune system (Verdonck et al., 2008; Devriendt et al., 2010a). In addition, these F4 fimbriae are potent mucosal immunogens, since they elicit a fast secretion of F4-specific secretory IgA (SIgA) at the intestinal tissues upon oral administration, protecting piglets against a challenge infection (Van den Broeck et al., 1999a,b; Verdonck, 2002, 2004a).

SIgA responses can be generated by both T cell-dependent and T cell-independent pathways (Cerutti, 2008). Recently, Th17 cells and their production of IL-17A and IL-21 have been implicated in the induction of SIgA directed against gut-dwelling pathogens (Cao et al., 2012; Hirota et al., 2013). In addition, these antigen-specific Th17 cells also suggested responsible for the selection and regional production of high antigen-specific IgA⁺ B cells in the germinal center (GC) (Milpied and McHeyzer-Williams, 2013). This ability to trigger SIgA responses explains their critical function in the host defense against extracellular pathogens such as *Candida albicans*, *Citrobacter rodentium*, *Salmonella typhimurium*, *Klebsiella pneumonia*, and *Giardia muris* (Conti et al., 2009; Khader et al., 2009; Mayuzumi et al., 2010; Chen et al., 2011; Dreesen et al., 2014). Effective immunity to pathogens requires T lymphocytes to be endowed with appropriate effector properties. In this context, naive CD4⁺ T cells differentiate into different effector cells and tailor their functions to the nature of the microbial threat.

Besides the classical Th1 and Th2 cells, at least two other CD4⁺ T effector lineages have been identified and described, including Th17 and regulatory T cells (Tregs) (Luckheeram et al., 2012; Zhu et al., 2010). In humans and mice, Th17 cells can be induced from naive CD4⁺ T cells with IL-6 and/or IL-21 in the combination of TGF- β , and mainly secrete IL-17A, IL-17F and IL-21 (Kom et al., 2009). IL-17 (also known as IL-17A) is the hallmark cytokine of Th17 cells and is the founding member of the IL-17 cytokine family, which consists of six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F (Jin and Dong, 2013; Iwakura et al., 2011). Among the IL-17 family members, IL-17F shares the highest sequence homology with IL-17A (Angkasekwinai and Dong, 2011). Although both cytokines can bind to the same receptors, regulate inflammatory responses and are involved in mucosal defense, they show a distinct binding affinity for these receptors and as such different roles in triggering immunity (Iwakura et al., 2011). IL-17E on the other hand triggers Th2 immunity and is involved in the clearance of helminths and allergy (Fallon et al., 2006; Herberth et al., 2010). Recently, IL-17C produced by goblet cells and enteroendocrine cells has been proposed to mediate the intestinal inflammation in IBD patients (Friedrich et al., 2015). The function of the other IL-17 cytokines in immunity is still poorly understood.

As in humans, porcine Th17 cells arise from naive CD4⁺ lymphocytes via IL-6 in the context of TGF- β and secrete IL-17A and IL-21 (Kiros et al., 2011). However, whether Th17 cells or IL-17 cytokine family members participate in the immune response against an ETEC infection in pigs or other species is still unclear. The heat labile toxin (LT) from a human ETEC strain was found to enhance IL-17A production by human PBMCs in response to antigen or mitogen stimulation (Leach et al., 2012). Similarly, the IL-17A promoting effect of LT was also reported in mice upon *Helicobacter pylori* infection (Sjökvist Ottsjö et al., 2013). In pigs, IL-17A mRNA production in the small intestine was upregulated early during F4⁺ ETEC infection (Loos et al., 2012). In contrast, serum IL-17A levels were unaltered early during ETEC infection, while in the intestinal tissues a downregulated IL-17A mRNA production 7 days post infection was observed (Zhu et al., 2014). However, as F4⁺ ETEC infections are usually cleared

within 7-8 days, we hypothesized that a potential Th17 response due to ETEC infection should occur earlier. Thus, in the present study, we addressed if an F4⁺ ETEC infection and oral administration with F4 fimbriae could skew the T helper cell differentiation to a Th17 profile by assessing the mRNA expression profile of key transcription factors and cytokines involved in T-cell polarization at systemic and intestinal tissues.

4.2 Materials and methods

The methodology of the animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2014/01).

4.2.1 F4⁺ ETEC challenge

Six F4 receptor-positive (F4R⁺) piglets (7-8 week-old, Belgian Landrace) were selected based on the MUC4 TaqMan assay as previously described (Nguyen et al., 2013). Upon arrival in the animal care facilities, all animals were treated orally with colistin (150 000 U/kg of body weight/day; Colivet; Prodivet Pharmaceuticals, Eynatten, Belgium) until three days before inoculation to prevent potential ETEC infections due to stress caused by transport and handling of the animals. During the whole trial all pigs had access to water and feed *ad libitum*. To reduce the bacterial gut flora, the piglets were given orally a broad-spectrum antibiotic florfenicol (4-5 mg/kg body weight, Nuflor; Schering-Plough, Brussels, Belgium) for each pig for two consecutive days. One day after antibiotic administration, piglets were inoculated with the ETEC reference strain GIS26 (O149:K91:F4ac⁺, LT⁺STa⁺STb⁺) or phosphate buffered saline (PBS) on day 0 (D0) and day 1 (D1). In brief, piglets were sedated with Stressnil (40 mg/mL; Janssen-Cilag, Berchem, Belgium) and the gastric pH was neutralized by intragastric administration of 60 mL NaHCO₃ (1.4% in distilled water) followed by intragastric administration of 10¹⁰ GIS26 in 10 mL sterile PBS. Faeces were collected at D0 and D1 and at D2, D3 and D4 post infection to determine F4⁺ ETEC shedding as previously described (Van den Broeck et al., 1999a). Briefly, fecal samples were first inoculated onto blood agar plates at 37°C for 24h. Then the haemolytic *E.coli* colonies were examined by dot blotting the colonies on nitrocellulose membranes and detecting F4 fimbriae by agglutination with F4ac-specific mAb. The severity of diarrhea was scored daily as previously described (Jamalludeen et al., 2009). On D4, piglets were euthanized using

pentobarbital (Kela NV, Belgium) and intestinal tissues were excised. Jejunal segments with and without Peyer's patches, ileal segments and mesenteric lymph nodes (MLNs) were collected and washed twice with Krebs-Henseleit buffer (0.12 M NaCl, 0.014 M KCl, 1 mM KH₂PO₄, 0.025 M NaHCO₃, pH 7.4) and once with Krebs-Henseleit buffer containing 1% (v/v) formaldehyde. Next, the MLNs and intestinal samples were frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

4.2.2 Immunohistochemistry

The intestinal tissues were sampled as described above, washed with Krebs-Henseleit buffer, embedded in methocel (Fluka, Bornem, Belgium), snap-frozen in liquid nitrogen and stored at -80 °C until sectioning. Cryosections (14 µm) were cut and mounted on 3-aminopropyl-triethoxysilane (Sigma-Aldrich, Bornem, Belgium)-coated glass slides. After drying for 30 min at room temperature (RT), the slides were fixed in 4% paraformaldehyde for 20 min at 4 °C and then embedded in 0.1% Triton (Triton™ X-100, Sigma-Aldrich) for 10 min at RT. Slides were washed 3 times with PBS with gentle agitation and then incubated overnight with anti-CD3 mAb (0.5 µg/mL, mouse IgG₁, clone PPT3) and biotinylated anti-swine IL-17A polyclonal rabbit antibody (2.5 µg/mL, Kingfisher biotech, St. Paul, MN, USA) in PBS at 4 °C in a humidified chamber. Purified Mouse IgG₁ (0.5 µg/mL, Life Technologies, Carlsbad, CA, USA) and irrelevant rabbit polyclonal IgG (2.5 µg/mL, ab27472, Abcam, Cambridge, UK) were used as negative control. The next day, the sections were washed and incubated with streptavidin-FITC (2.5 µg/mL, Biolegend, London, UK) and Texas Red-X conjugated goat anti-mouse IgG (H+L) secondary antibody (5 µg/mL, Life Technologies) at RT for 1.5 h. Subsequently, the sections were washed in PBS and then the nuclei were counterstained with Hoechst (10 µg/mL, Sigma-Aldrich) for 15 min at RT. Finally, the slides were mounted in glycerol containing 0.223 M 1,4-diazobicyclo-(2,2,2)-octane (Sigma-Aldrich) and imaged on a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH).

4.2.3 Isolation of peripheral blood mononuclear cells

Blood was taken on heparin from the jugular vein of piglets on D0 and D1 prior to inoculation and on D2, D3 and D4 post infection. PBMCs were isolated by density gradient centrifugation using Lymphoprep (Axis-shield, Oslo, Norway). Erythrocytes were lysed in ammonium chloride solution. The resulting PBMC fraction was washed twice in ice cold PBS + 1 mM ethylenediaminetetraacetic acid (EDTA) and counted using a hemocytometer. The viability was confirmed by exclusion of the vital dye Trypan blue. Then, the cells were either put in TRIzol Reagent (1×10^7 cells in 1 mL TRIzol Reagent; Life Technologies) for RNA extraction or cultured at a concentration of 5×10^6 cells/mL in leukocyte medium (RPMI-1640 (Gibco), fetal bovine serum (FBS) (10%) (Gibco), sodium pyruvate (1 mM) (Gibco), L-glutamine (2 mM) (Gibco), penicillin (100 IU/mL), streptomycin (100 µg/mL), (Gibco), and non-essential amino acids (1%)) in the absence or the presence of F4 fimbriae (5 µg/mL) for up to 72 h at 37 °C, 5% CO₂ in a humidified atmosphere. Next, the cells were collected, lysed in 1 mL TRIzol and stored at -80 °C until RNA extraction. Cell supernatants were collected and stored at -80 °C until further processing.

4.2.4 RNA extraction and cDNA synthesis

Prior to RNA extraction, frozen tissue sample were homogenized in liquid nitrogen with mortar and pestle. RNases were removed by baking mortar and pestle at least 3 h at 200 °C. Briefly, mortar and pestle were chilled in liquid nitrogen followed by grinding of the tissues until a fine powder was formed. This powder (600-1200 mg) was added to 1.0 mL prewarmed (37 °C) TRIzol Reagent and immediately mixed well. Then RNA was extracted following the manufacturer's instructions. RNA samples were treated with DNase I (Promega, Madison, WI, USA) and purified with the RNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands) according to the manufacturer's guidelines. The RNA concentration and purity were determined by measuring the optical density at OD₂₆₀/OD₂₈₀ and OD₂₆₀/OD₂₃₀ with a NanoDrop 2000/2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples had OD₂₆₀/OD₂₈₀ ratios between 1.9 and 2.0 and OD₂₆₀/OD₂₃₀ ratios between 1.9 and 2.1. Total RNA (1 µg) was reverse transcribed using SuperscriptTM III Reverse Transcriptase (200 U; Life Technologies) and random primers (7.5 ng/µL; Life Technologies). To check the

synthesis of amplifiable cDNA in the reverse transcription, a conventional PCR step was performed using GAPDH and β -actin specific primers (**Table 1**).

4.2.5 Real-time qPCR

Primers (**Table 1**) were designed using Primer 5 to span an exon-exon junction thereby avoiding amplification of genomic DNA. The primers were purchased from Eurogentec (Liege, Belgium). The amplification efficiency of all the reactions ranged from 94% to 103%. The PCR products were sequenced and subjected to agarose gel electrophoresis to verify their specificity. cDNA was diluted 8x in DEPC-treated ddH₂O and combined with primer pairs and SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) according to the manufacturer's recommendations. Quantitative PCR (qPCR) assays were run on the StepOnePlus real-time PCR system (Applied Biosystems) with the following cycling conditions: 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing for 30 s and elongation at 72 °C for 30 s. Fluorescence acquisition was measured at 72 °C and melting curve analysis was done at 65-95 °C with continuous fluorescence acquisition. The stability of the GAPDH, β -actin, 60S ribosomal protein L19 (RPL-19) and Cyclophilin A (CyPA) mRNA expression levels was evaluated by geNorm (Vandesompele et al., 2002). We finally selected GAPDH, β -actin and RPL-19 as reference genes. All reactions were performed in triplicate and relative gene transcription values were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized against these three selected reference genes (Livak et al., 2001).

Table 1. List of the primers used in the qPCR assay.

Gene	Sequence (5'→3')	size (bp)	Ta (°C)	Reference
IL-17A	F: ACTCCAAACGCTTCACCTCAC	234	58	NM_001005729.1
	R: AGCCCACTGTCACCATCACTT			
IL-17B-like	F: CTGGCCAAGAGGAAGTGTGAG	92	60	XM_003124086.1
	R: GGGTCGTGGTTGATGCTGTAG			
IL-17F	F: GAGGCAGCAGCTCGGAAAAT	173	60	NC_010449.4
	R: TCCCGGGTGATGTTGTAATCC			
IL-21	F: GGCACAGTGGCCCATAAATC	124	60	Kiros et al., 2011
	R: GCAGCAATTCAGGGTCCAAG			
IL-22	F: TTGACCAGTCCAACTTCCAGCAGC	143	60	XM_001926156.1
	R: GCAGCGCTCTCTCATATTGACTCC			
IL-23p19	F: CCAAGAGAAGAGGGAGATGATGA	107	57	NM_001130236.1
	R: TGCAAGCAGGACTGACTGTTGT			
RORγt	F: TTCAGTACGTGGTGGAGTTC	141	60	Kiros et al., 2011
	R: TGTGGTTGTCAGCGTTGTAG			
IL-10	F : CCATGCCCAGCTCAGCACTG	295	60	Melkebeek et al., 2007
	R: CCCATCACTCTCTGCCTTCGG			
IL-13	F: GTCATTGCTCTCACCTGCTT	308	58	Raymond and Wilkie 2004
	R: TTGGTGTCTCGGATGTGCTT			
GATA-3	F: GCTCTACCACAAAATGAACGGAC	110	58	NM_001044567.1
	R: TCGTTGTGGTTTGACAGTTTGC			
IL-12p40	F: GGTTTCAGACCCGACGAACCTCT	112	60	Devriendt et al., 2009
	R: CATATGGCCACAATGGGAGATG			
IFN-γ	F: GAGCCAAATTGTCTCCTTCTACT	262	60	Verhelst et al., 2011
	R: CTGACTTCTCTCCGCTTTCT			
T-bet	F: TCAATCCTACTGCCCCTAC	151	60	Pilon et al., 2009
	R: TTAGGAGACTCTGGGTGAAC			
Foxp3	F: TGCCATTGCCCACAACCTT	179	60	NM_001128438.1
	R: CCTGTCCATCCTTCTTTCTT			
AID	F: AGAAGTTTCAAAGCCTGGGAG	92	57	XM_003126511.1
	R: TCAACCTCATACAGGGGCAAA			
β-actin	F: TCATCACCATCGGCAACG	133	60	von der Hardt et al., 2004
	R: TTCCTGATGTCCACGTCGC			
GAPDH	F: GGGCATGAACCATGAGAAGT	230	60	Melkebeek et al., 2007
	R: AAGCAGGGATGATGTTCTGG			
RPL19	F: AACTCCCGTCAGCAGATCC	147	60	Pilon et al., 2009
	R: AGTACCCTTCCGCTTACCG			

Abbreviations: RORγt = RAR-related orphan receptor gamma t, AID = Activation-induced (cytidine) deaminase, RPL-19 = 60S ribosomal protein L19

4.2.6 Purification of F4 fimbriae

F4 fimbriae were purified from the ETEC reference strain GIS26 or the IMM01 strain (O147:F4ac⁺LT⁺STb⁺, which lacks flagellin expression) as previously described (Van den Broeck et al., 1999a). Briefly, the bacteria were cultured in tryptone soy broth (Difco Laboratories, Biotrading, Bierbeek, Belgium) at 37 °C for 18 h, collected by centrifugation and washed in sterile PBS. Subsequently, F4 fimbriae were isolated by mechanical shearing of the bacterial suspension followed by centrifugation to remove the remaining bacteria. The fimbriae were precipitated with ammonium sulfate (40% saturation), the pellet was dissolved in PBS and dialysed overnight against PBS at 4 °C. Next, the fimbrial proteins were filtrated (0.22 µm) and the endotoxins were removed by using EndoTrap columns (Hyglos GmbH, Regensburg, Germany) following the manufacturer's guidelines. After endotoxin removal, the fimbrial solution contained almost no endotoxin (0.24 EU/mL) as determined by the Limulus amoebocyte lysate test (Lonza, Walkersville, MD, USA). The protein concentration was determined by the bicinchoninic acid reaction (Sigma-Aldrich) with bovine serum albumin as a standard and the purity was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 12%).

4.2.7 Oral immunization with purified F4 fimbriae

Eleven F4 receptor-positive (F4R⁺) piglets (4-5 weeks old) were selected, housed and treated with antibiotics as described above. The experimental group consisted of six F4 fimbriae seronegative pigs (three Hypor-west and three Large White × Belgian Landrace), while the control group contained five F4 fimbriae seropositive pigs (two Hypor × Pietrain and three Yorkshire × Large White × Landrace). Prior to the oral immunization piglets were deprived of feed and water for 3 h. Purified F4 fimbriae from the GIS26 strain (1 mg in 10 mL PBS) or PBS were administered orally for three subsequent days to the piglets of the experimental or the control group, respectively. Blood was taken from the jugular vein on the day prior to the initial immunization and at D4 and D9 post immunization to isolate PBMCs for RNA extraction as described above. Blood samples of D0 and D9 were also used to measure F4-specific serum IgG and IgA antibodies by ELISA to monitor immunization success (Van den Broeck et al., 1999a).

4.2.8 *In vitro* culture of PBMCs

Blood was taken from 8-12 week-old healthy conventionally reared, F4 seronegative pigs (Belgian Landrace) and PBMCs were isolated and suspended at a concentration of 5×10^6 cells/mL in leukocyte medium as described above. Subsequently, PBMCs were transferred to a 24-well tissue culture plate and stimulated with 5 µg/mL F4 fimbriae in the presence or absence of polymyxin B (Sigma-Aldrich), endotoxin-free F4 fimbriae (5 µg/mL) or medium at 37 °C, 5% CO₂ in a humidified atmosphere. The cells and supernatants were harvested at 24 h, 48 h and 72 h after stimulation and stored properly as mentioned above. The mRNA expression profile of the PBMCs was analyzed using qPCR as described above.

4.2.9 Cytokine ELISA

The secretion of IL-17A, IL-10, IFN-γ and IL-22 in cell-free supernatants was measured using commercial ELISA kits according to the manufacturer's guidelines (IL-17A and IFN-γ, Kingfisher biotech; IL-10, Life Technologies; IL-22, Sigma-Aldrich).

4.2.10 Statistical analysis

Statistical analysis was performed with the Mann-Whitney U test or Kruskal-Wallis Test for the independent samples and Friedman's Two-Way Analysis for the related samples in the SPSS 22 software package. The significance level was set at $p < 0.05$.

4.3 Results

4.3.1 F4⁺ ETEC infection triggers IL-17 signature responses in PBMCs and small intestinal tissues

To analyze the type of immune response elicited by F4⁺ ETEC, we assessed the mRNA expression profile of key cytokines and transcription factors involved in either T cell polarization or their effector functions. Indeed, the mRNA expression levels of Th1 (IFN-γ, IL-12, T-bet), Th2 (IL-13, GATA-3), Th17 (IL-17A, IL-21, IL-22 IL-23p19, orphan nuclear receptor (RORγt)) and regulatory T cells (Foxp3, IL-10) were evaluated in PBMCs and several intestinal tissues. In addition, the mRNA expression of the IL-17 family cytokines IL-17B and IL-17F as well as activation-induced cytidine deaminase (AID), a B-cell specific enzyme required for somatic hypermutation and class switch recombination, was assessed. Following F4⁺ ETEC challenge, only one of the three pigs exhibited severe diarrhea, while F4⁺ ETEC shedding was detected in all infected

pigs (**Table 2**). Notably, the expression of IL-17A mRNA was significantly increased in PBMCs at D2 and D3 after F4⁺ ETEC infection (**Figure 1**). In addition, an increased mRNA expression of IL-23p19 and ROR γ t was also detected these days (**Figure 1**). Moreover, F4⁺ ETEC infection also increased the mRNA expression of the Th17 cytokines IL-21, IL-22 and IL-17F. In contrast, IL-17B mRNA expression was downregulated in F4⁺ ETEC infected pigs (**Figure 1**). With regard to the Th1-related genes, only the mRNA expression of IFN- γ was upregulated at D3 and D4, while the mRNA expression of the Th1-related transcription factor T-bet and the Th1-inducing cytokine IL-12 was not influenced by F4⁺ ETEC infection. ETEC infection also significantly enhanced the mRNA expression of the Th2-related transcription factor GATA-3 and the Tregs-related genes Foxp3 and IL-10. Also the AID mRNA levels increased by F4⁺ ETEC in a time-dependent manner in the PBMCs compared to control pigs, which could indicate the presence of pathogen-specific circulating B cells undergoing class switching.

Table 2 Fecal excretion of hemolytic F4⁺ETEC after oral challenge

Mean F4 ⁺ ETEC Number (/g faeces)						
	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6
Day 4	0	20	0	3x10 ⁵	4x10 ⁶	2x10 ⁴
Day 3	0	0	0	3.4x10 ⁷	1.6x10 ⁵	1.7x10 ⁵
Day 2	0	0	0	5.5x10 ⁷	3.2x10 ⁷	1x10 ⁷
Day 1	0	0	0	3x10 ⁶	5.7x10 ⁶	2.2x10 ⁶
Day 0	0	10	0	0	0	0

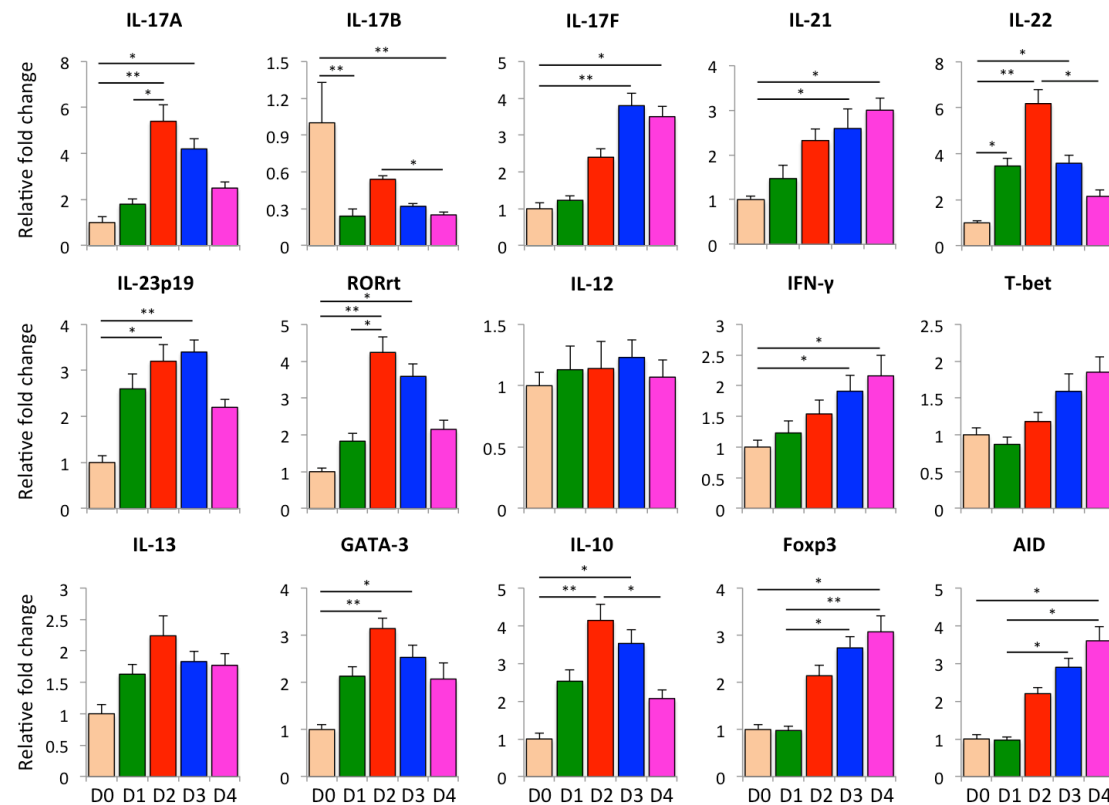


Figure 1 The mRNA expression profile in PBMCs triggered by F4⁺ ETEC infection. Piglets were infected with F4⁺ ETEC on day 0 (D0) and day 1 (D1). PBMCs were isolated from piglets on D0 until D4 after infection. The mRNA expression in the PBMCs of F4⁺ ETEC infected and control piglets was analyzed by qPCR. The mRNA expression level was normalized to the reference genes and then to the control group for every separate day. Then, the data were plotted relative to D0. Data are presented as the mean ± SEM (*n* = 3 per group). * *p* < 0.05, ** *p* < 0.01.

We further compared the expression profiles of these transcription factors and cytokines in several intestinal tissues on D4 after F4⁺ ETEC infection. As shown in **Figure 2**, the fold changes of almost all the examined transcripts were higher in the Peyer's patches (PP) and MLNs than those in the jejunal and ileal lamina propria after F4⁺ ETEC infection. Interestingly, these transcripts also displayed a higher expression level in the PP and MLN of control pigs (**Figure 3**). Similar to PBMCs, F4⁺ ETEC infection significantly increased IL-17A, IL-17F, IL-21 and IL-23p19 mRNA levels in all examined tissues as compared to control pigs (**Figure 2**). In addition, IL-17A, IL-17F and IL-23p19 transcripts were strongly induced in PP and MLN. In contrast to PBMCs, IL-17B mRNA expression was upregulated upon F4⁺ ETEC infection in all intestinal tissues except jejunum. No significant difference in IL-22 mRNA expression was observed in jejunal and ileal lamina propria, whereas a small upregulation was found in the ileal PP and MLN in F4⁺ ETEC infected pigs as compared to control pigs. Regarding the Th2-related genes, we observed upregulated IL-13 and GATA-3 mRNA levels with the highest change in the ileal PP and MLN, respectively. Similar to the systemic immune system, F4⁺ ETEC infection did not alter the intestinal mRNA expression of IL-12, IFN- γ and T-bet (**Figure 2**), while both Foxp3 and IL-10 mRNA levels were significantly upregulated, especially in the PP and MLN. Interestingly, mRNA expression of AID was highly upregulated upon F4⁺ ETEC infection in the PP and MLN, indicating active class switching in those tissues.

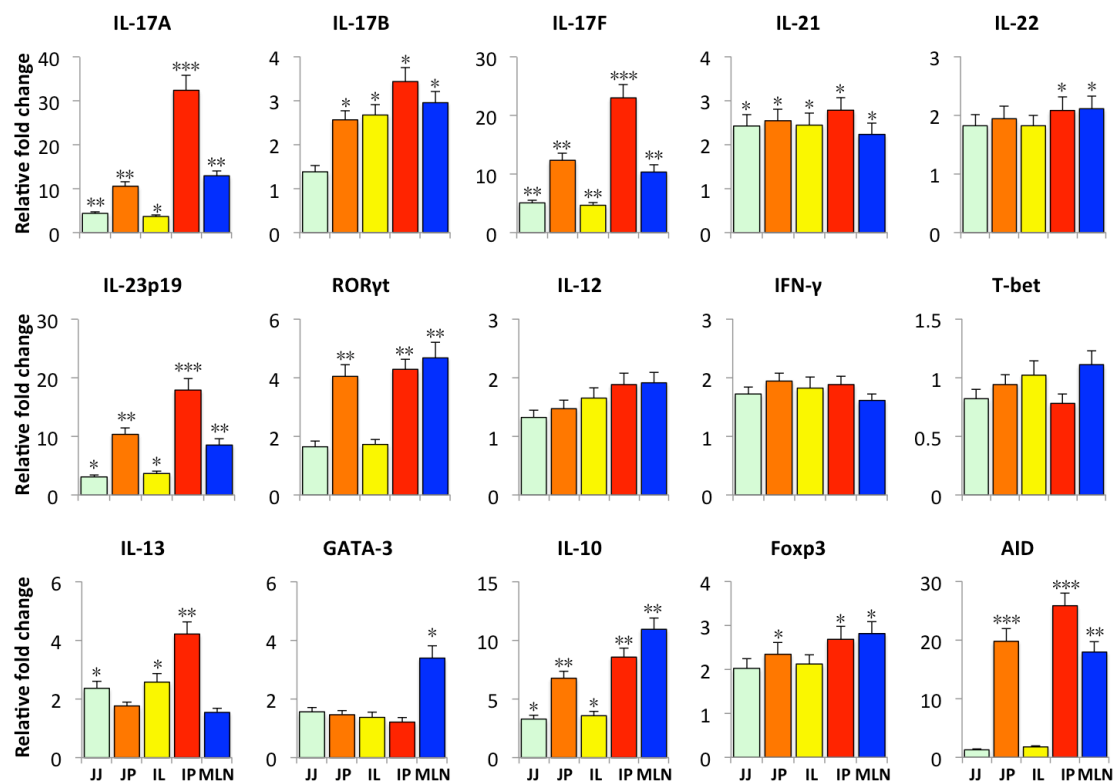


Figure 2 F4⁺ ETEC infection induced mRNA expression profile in intestinal tissues. The F4⁺ ETEC infection was performed on day 0 (D0) and day 1 (D1). Intestinal samples were collected on D4. The mRNA expression in intestinal tissues of F4⁺ ETEC infected or control pigs was analyzed by qPCR. The mRNA expression was normalized to the reference genes and then to the control group for all separate intestinal tissues. Data are presented as the mean \pm SEM (n = 3 per group). JJ = jejunum without Peyer's patches, JP = jejunum with Peyer's patches, IL = ileum without Peyer's patches, IP = ileum with Peyer's patches, MLN = mesenteric lymph nodes. * indicates significant differences as compared to the control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

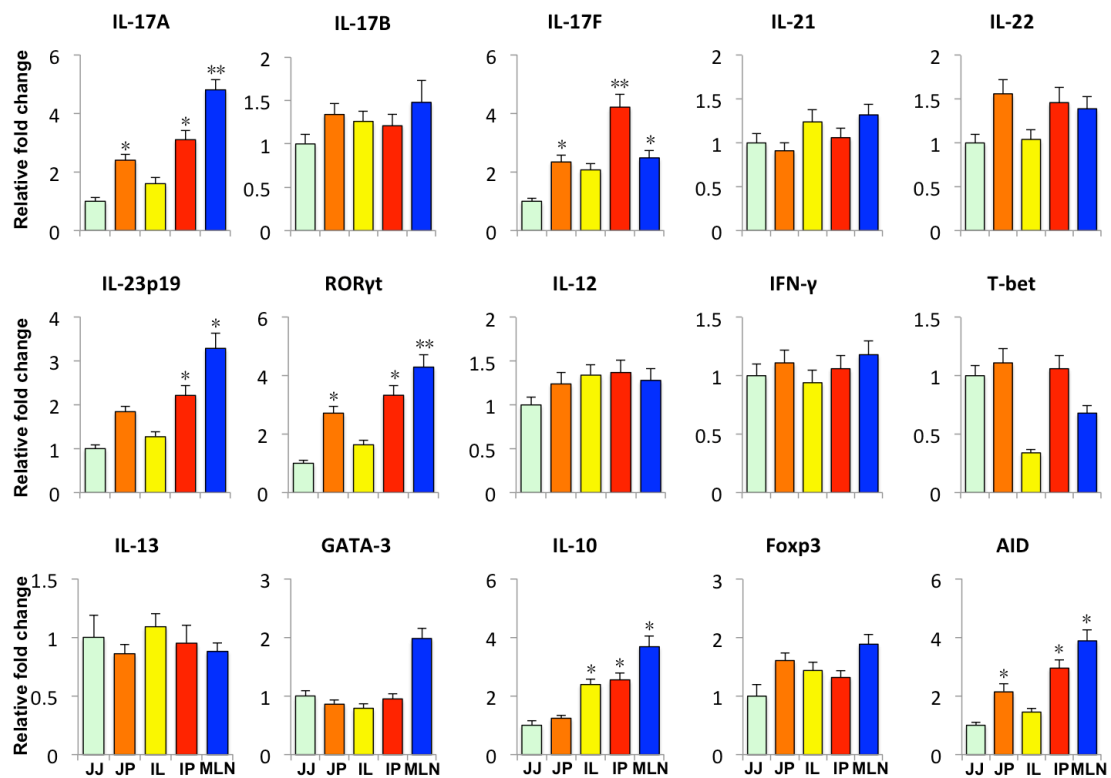


Figure 3 The distribution of mRNA in intestinal tissues of control pigs. The mRNA expression in intestinal tissues of control pigs was analyzed by qPCR. The mRNA expression was normalized to the reference genes and then to jejunum without Peyer's patches. Data are presented as the mean \pm SEM ($n = 3$ per group). JJ = jejunum without Peyer's patches, JP = jejunum with Peyer's patches, IL = ileum without Peyer's patches, IP = ileum with Peyer's patches, MLN = mesenteric lymph nodes. * $p < 0.05$, ** $p < 0.01$.

4.3.2 F4⁺ ETEC infection increased CD3⁺IL-17A⁺ T cells in the intestinal tissue

Given the high expression of IL-17A mRNA in the ileal PP of F4⁺ ETEC infected piglets, we assessed if this correlated with an influx of IL-17A⁺ T cells in that tissue. Immunofluorescence analysis of ileal tissue clearly showed an increase in CD3⁺IL-17A⁺ cells in the crypts and villi in F4⁺ ETEC infected piglets as compared to controls. In contrast, colocalization of CD3 and IL-17A was rarely observed in the tissue of control pigs (**Figure 4**).

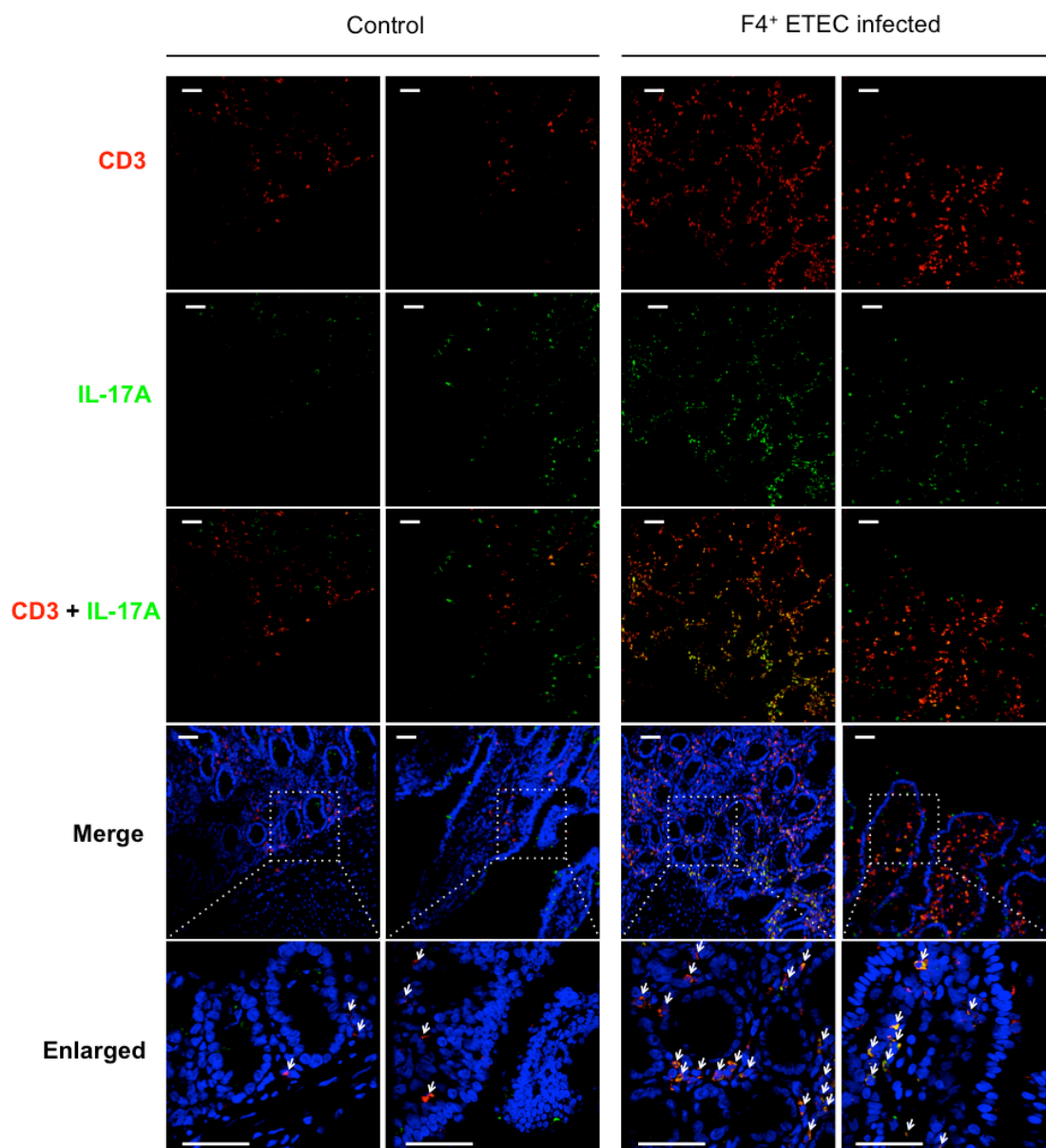


Figure 4 Increased presence of IL-17A⁺ T cells in the ileum of F4⁺ETEC infected piglets. Cryosections were stained with anti-IL-17A (FITC, green) and anti-CD3 mAbs (Texas Red-X, red). The nuclei were counterstained with Hoechst (blue). Images are representative for all piglets in both groups. The arrows indicate colocalization of CD3 and IL-17A.

4.3.3 Oral immunization of piglets with F4 fimbriae triggered systemic IL-17 responses

We detected robust Th17 and IL-17-related cytokine responses upon F4⁺ ETEC infection both in intestinal tissues and PBMCs. In a next effort we wanted to determine if these observations could be reproduced by oral immunization of piglets with F4 fimbriae, as these are potent oral immunogens. We only focused on the PBMCs since similar systemic and mucosal responses were found after F4⁺ ETEC infection. Upon immunization, the F4-specific IgG and IgA serum antibody titers were significantly increased as compared to the control group (**Figure 5**), indicating a successful immunization. Similar to F4⁺ ETEC infection, oral immunization with F4 fimbriae significantly increased the mRNA expression of Th17-related genes, including IL-17F, IL-21, IL-22, IL-23p19 and ROR γ t, although with different kinetics (**Figure 6**). Compared to control pigs, significant changes were only observed at D4 for IL-17F and IL-23p19 and at D9 for IL-22, whereas IL-21 and ROR γ t mRNA expression levels peaked at D4. Unexpectedly, IL-17A mRNA expression was undetectable in porcine PBMCs upon oral immunization with F4 fimbriae. In contrast to the PBMCs of F4⁺ ETEC infected pigs, oral immunization with F4 fimbriae upregulated IL-17B mRNA expression at D4. In agreement with F4⁺ ETEC infection, only IFN- γ , but not IL-12 and T-bet, mRNA expression was elevated in immunized pigs. Likewise, GATA-3 levels increased in a time-dependent manner to reach significance at D9. Unexpectedly, we also failed to detect IL-13 mRNA expression in PBMCs upon F4 fimbriae immunization. Regarding the Tregs-related genes, only Foxp3 mRNA expression was enhanced. Similar to the infection trial, we also found a significant increase of AID mRNA expression, presumably indicating the presence of F4-specific circulating B-cells undergoing class switching.

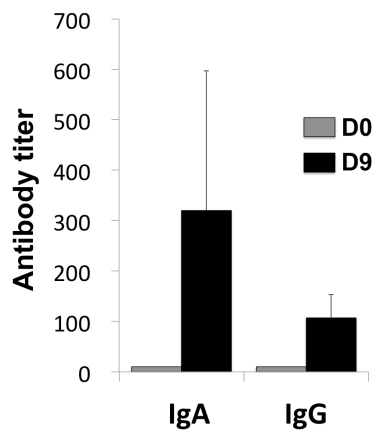


Figure 5 Oral immunization with F4 fimbriae induced F4-specific serum antibodies. The piglets were immunized with 1 mg F4 fimbriae on day 0 (D0), D1 and D2. Blood was drawn on D0 before immunization and D9 after immunization. The F4-specific antibody (Ab) titer was tested in serum using ELISA. Data are presented as the mean \pm SEM ($n = 5$ per group).

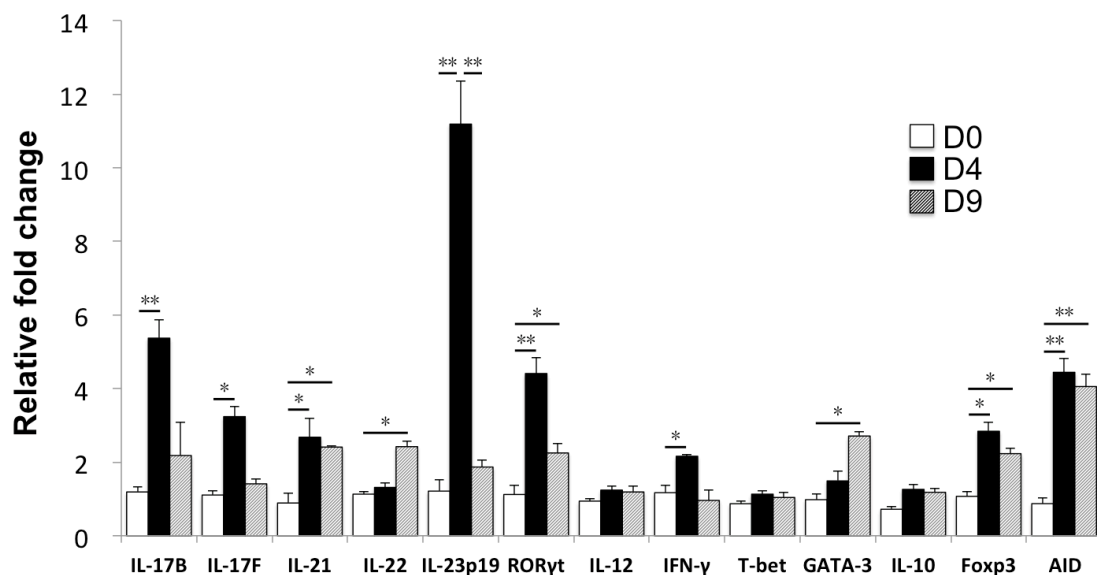


Figure 6 F4 fimbriae trigger mRNA expression of IL-17 cytokines in PBMCs upon oral immunization. The piglets were immunized with 1 mg F4 fimbriae on day 0 (D0), D1 and D2. PBMCs were isolated from piglets on D0 before immunization and on D4 and D9 after immunization. The mRNA expression in PBMCs isolated from F4-immunized or control piglets was analyzed by qPCR. The mRNA expression level was normalized to the reference genes and then to the control group for every separate day. Data are presented as the mean \pm SEM ($n = 5$ per group). * $p < 0.05$, ** $p < 0.01$.

4.3.4 F4 fimbriae induced a Th17-signature cytokine expression in naive PBMCs

The above data indicate the potential of F4 fimbriae to induce Th17 responses. To further address this potential, PBMCs were isolated from naive animals and stimulated with F4 fimbriae. Upon stimulation for 24 h the mRNA expression levels of IL-17A, IL-17F and IL-22 were significantly increased as compared to unstimulated cells (**Figure 7**). This upregulated expression level lasted at least till 72 h post stimulation. In addition, F4 fimbriae significantly enhanced IL-23p19 mRNA expression, which peaked at 24 h post stimulation, while IL-21 and ROR γ t mRNA expression only displayed a significant change at 72 h (**Figure 7**). Intriguingly, F4 fimbriae stimulated PBMCs downregulated IL-17B mRNA expression. Regarding the Th1-related genes, we only observed a significant upregulation of IFN- γ transcripts, while IL-12 and T-bet mRNA levels were not affected. Furthermore, no significant difference in the mRNA expression of Foxp3, IL-10, GATA-3 and IL-13 was observed. Similar to the infection and immunization experiment, AID mRNA levels were significantly increased in F4 fimbriae stimulated PBMCs (**Figure 7**).

To confirm that the increased transcript levels upon stimulation of PBMCs with F4 fimbriae resulted in higher protein levels, we measured the secretion of the corresponding cytokines using ELISA. As shown in **Figure 8**, stimulation with F4 fimbriae significantly increased IL-17A secretion by PBMCs as fast as 24 h post stimulation. Moreover, endotoxins in the fimbrial solution did not exert a significant effect on the IL-17A secretion (**Figure 9**). F4 fimbriae also triggered the secretion of IL-22 by PBMCs, while no significant changes were observed for IL-10 and IFN- γ .

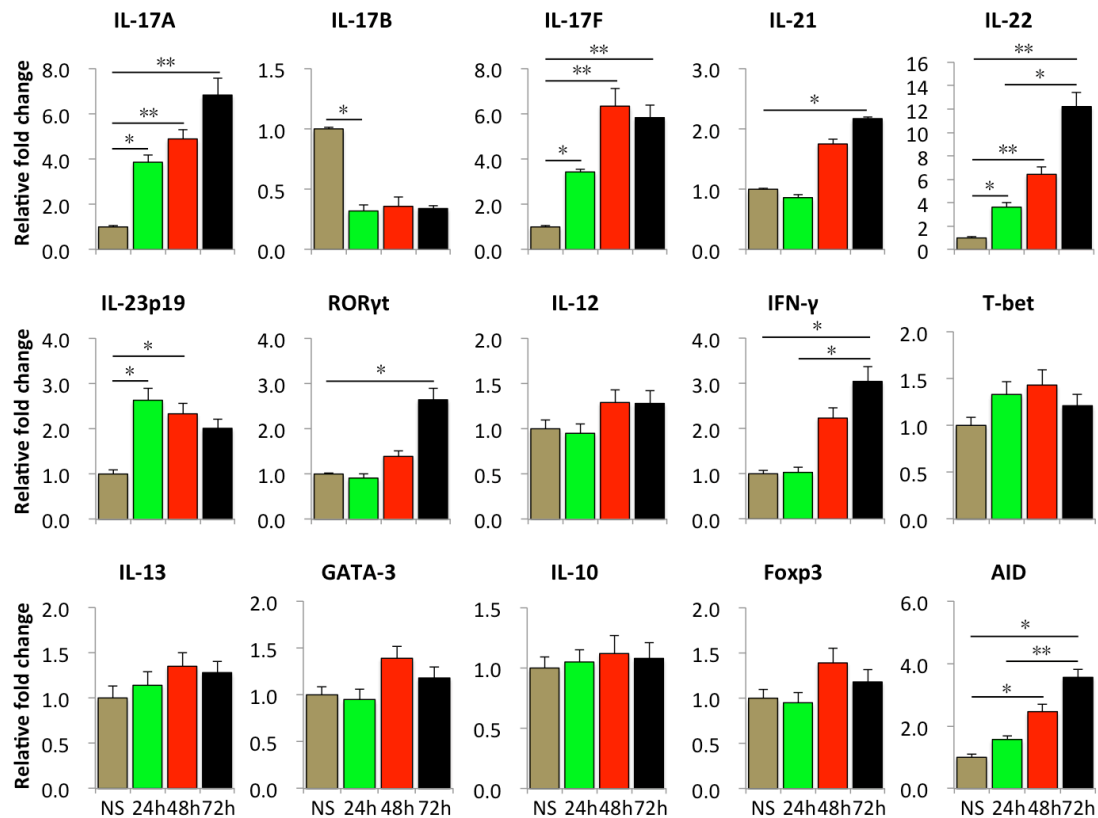


Figure 7 F4 fimbriae elicit an IL-17 dominated cytokine response in naïve PBMCs. PBMCs were isolated from naïve piglets and were stimulated with F4 fimbriae (5 µg/mL) for 24, 48 and 72 h. The mRNA expression profile was analyzed by qPCR. mRNA expression levels were normalized to the reference genes and then to the control group for each time point. Then, the data for every day was plotted relative to day 0. Data are presented as the mean ± SEM (*n* = 3). NS = non-stimulated. * *p* < 0.05, ** *p* < 0.01.

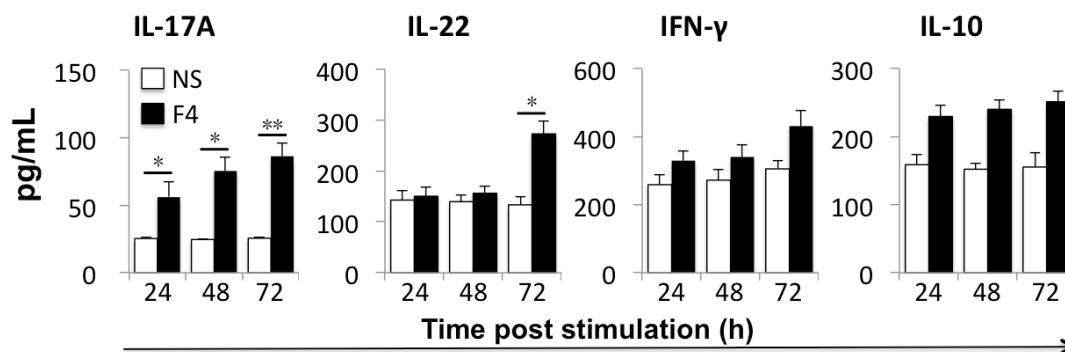


Figure 8 Cytokine secretion by F4 fimbriae stimulated naïve PBMCs. PBMCs were stimulated with endotoxin-free F4 fimbriae (5 µg/mL) or medium for 72 h. The protein level of IL-17A, IL-22, IFN-γ and IL-10 in the supernatant was determined by ELISA. Data are presented as the mean ± SEM (*n* = 3). NS = non-stimulated. * *p* < 0.05, ** *p* < 0.01.

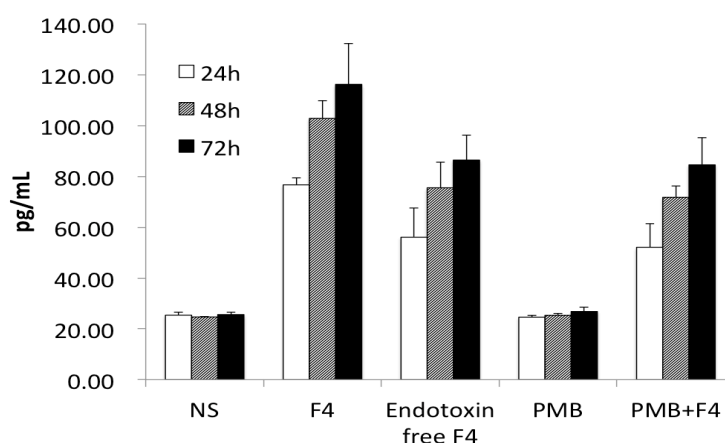


Figure 9 Effect of endotoxins on IL-17A secretion. PBMCs were stimulated with endotoxin-free F4 fimbriae (5 µg/mL), F4 fimbriae (5 µg /mL) in the presence or absence of polymyxin B (PMB, 25 µg /mL) or medium for 72 h. The IL-17A concentration in the cell-free supernatant was determined by ELISA. Data are presented as the mean \pm SEM ($n = 3$). NS = non-stimulated.

4.3.5 F4 fimbriae boosted Th17 responses in an antigen recall assay

To confirm the presence of circulating F4-specific lymphocytes, PBMCs from F4⁺ ETEC infected pigs were restimulated with F4 fimbriae for 48 h and the mRNA expression levels of the above mentioned cytokines and transcription factors were assessed. As shown in **Figure 10A**, restimulation with the fimbriae resulted in the upregulation of all examined transcripts, except IL-13 and IL-12. The Th17-related genes IL-17A, IL-17B, IL-17F, ROR γ t, IL-21, IL-22 and IL-23p19 as well as AID were highly upregulated. In addition, Foxp3, IL-10, GATA-3, IFN- γ and T-bet mRNA expression levels were increased after F4 restimulation, although not to the same extent as the Th17-related cytokines. Furthermore, the upregulated IL-17A and IL-22 transcripts elicited by F4 fimbriae restimulation correlated with an augmented IL-17A and IL-22 cytokine secretion by the PBMCs, while the increase in IFN- γ and IL-10 production was not statistically significant (**Figure 10B**).

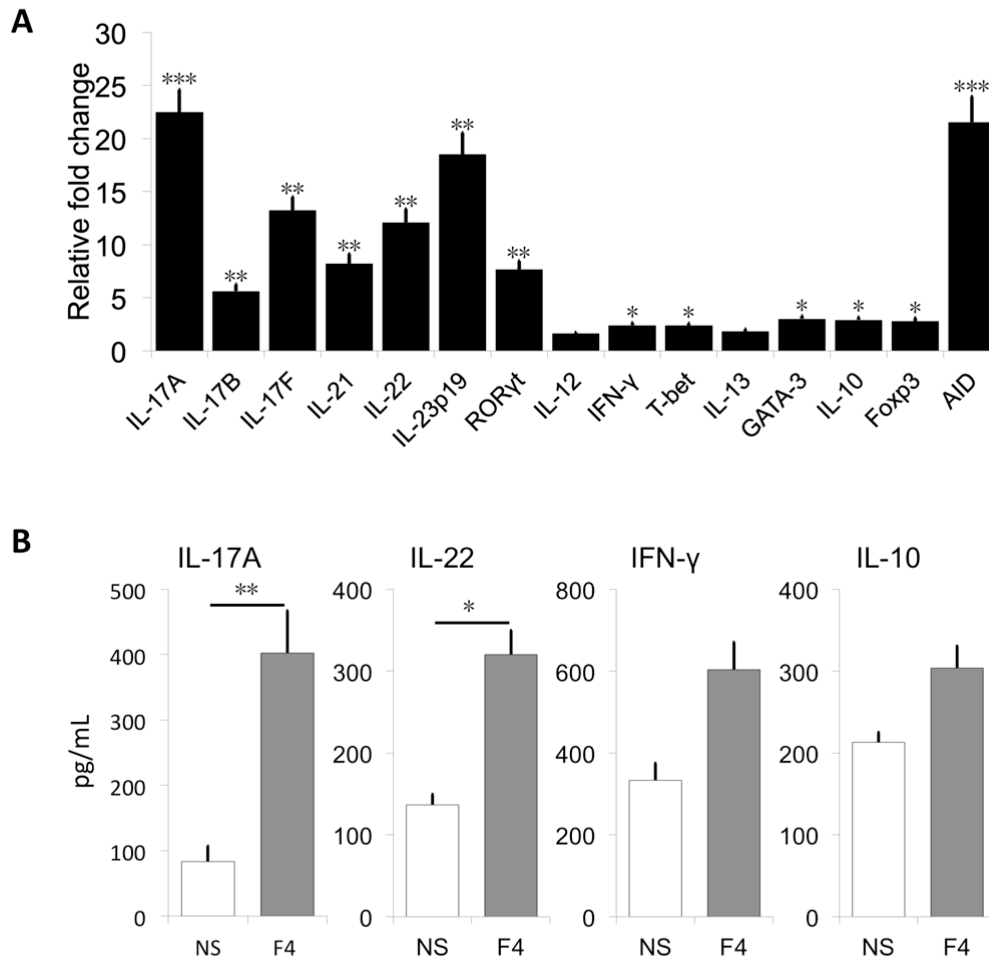


Figure 10 Th17 signature dominates in an antigen recall assay. PBMCs were isolated from F4⁺ ETEC infected animals and stimulated with F4 fimbriae (5 µg/mL) or medium for 48 h. **(A)** The mRNA expression profile in PBMCs was analyzed by qPCR. The mRNA expression level was normalized to the reference genes and then to control PBMCs. * indicates significant differences as compared to the control group. **(B)** The secretion of IL-17A, IL-22, IFN-γ and IL-10 by PBMCs was determined by ELISA. Data are presented as mean ± SEM (*n* = 3 per group). NS = non-stimulated. * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001.

4.4 Discussion

Th17 effector cells are important to eradicate mucosal pathogens including extracellular bacteria, fungi and even helminths (Khader et al., 2009; Blaschitz and Raffatellu, 2010; Bouchery et al., 2014). These Th17 cells are characterized by the secretion of IL-17A, IL-17F, IL-21 and IL-22. The latter contribute to the protective function of Th17 cells by inducing the expression of defensins, mucins, tight junction proteins and lipopolysaccharide-binding proteins, which all reinforce the epithelial barrier (Bogaert et al., 2010). Although some progress has been made to elucidate the cytokine response of intestinal epithelial cells to ETEC or its virulence factors (Pavlova et al., 2008; Devriendt et al., 2010b; Geens et al., 2010; Zanello et al., 2011a, b; Zhou et al., 2012), only few studies have evaluated the transcriptomic profile in PBMCs and intestinal tissues upon F4⁺ ETEC infection or oral immunization with F4 fimbriae prior to this report (Delisle et al., 2012). Recent work at our laboratory first hinted at the importance of IL-17A in innate immunity targeted to fend off F4⁺ ETEC infection as IL-17A mRNA levels were upregulated in the small intestine of piglets 4 h after F4⁺ ETEC colonization (Loos et al., 2012). Zhu and colleagues on the other hand observed a downregulated expression of IL-17A mRNA in both jejunum and ileum one week after F4⁺ ETEC challenge, although this downregulation did not reach the significant level. In addition, no significant changes were observed in the mRNA expression levels of IFN- γ , IL-12p40, IL-4, IL-2, IL-10, Foxp3 and TGF- β , except for an upregulated IL-6 mRNA expression (Zhu et al., 2014). As F4⁺ ETEC colonize the gut very soon upon ingestion and clearance of this pathogen usually occurs at 7 days post infection (Verdonck et al., 2002), any changes in cytokine expression levels should have occurred earlier. Here, we evaluated the mRNA expression profile in PBMCs from day 0 to 4 upon F4⁺ ETEC infection and at day 4 in the intestinal tissues. F4⁺ ETEC infection triggered an increased expression of IL-17A, IL-17F, IL-21, IL-22, IL-23p19 and ROR γ t in the intestine and PBMC fraction, hinting at a potential role of Th17 cells to clear F4⁺ ETEC infections in piglets. Indeed, IL-17A, IL-17F, IL-21 and IL-22 are preferentially produced by Th17 cells, while the transcription factor ROR γ t and IL-23 play essential roles in the differentiation and expansion of Th17 cells, respectively (Ouyang et al., 2008; McGeachy et al., 2008). The involvement of Th17 cells is also evidenced by the

occurrence of relatively large amounts of IL-17A producing T cells in the intestinal tissue of F4⁺ ETEC infected piglets. Interestingly, we also observed a strong upregulation of IL-17F mRNA expression. IL-17A and IL-17F have similar biological actions as both cytokines mediate pro-inflammatory responses and play a role in the host defense against certain mucosal pathogens, such as *C. rodentium* (Chang and Dong, 2009; Sabat et al., 2013; Kolls and Lindén, 2004). Thus, we speculate that both IL-17A and IL-17F are required for the protection against F4⁺ ETEC infection in piglets. Further research should elucidate the contribution of each cytokine to protection against ETEC, especially as divergent roles for IL-17A and -F in immunity have been reported (Yang et al., 2008; Ishigame et al., 2009). Intriguingly, we observed a differential regulation of IL-17B mRNA expression in intestinal tissues and PBMCs upon F4⁺ ETEC infection, which may suggest IL-17B has a different function in mucosal and systemic immunity. Not much is known about IL-17B. This cytokine is expressed by monocytes and neutrophils and induces the secretion of pro-inflammatory cytokines (Li et al., 2000; Moore et al., 2002; Kokubu et al., 2008; Kouri et al., 2014). Further research should identify the IL-17B-producing cells in pigs and elucidate their role in the host defense against mucosal pathogens.

In contrast to the increased level of Th17-related cytokines, F4⁺ ETEC infection did not affect the expression level of the Th1-related genes IL-12, IFN- γ and T-bet in both PBMCs and intestinal tissues, although a small increased IFN- γ expression was observed in the PBMC fraction on D3 and 4 upon infection. F4⁺ ETEC infection also resulted in an increased mRNA expression of IL-13, GATA-3, IL-10, and Foxp3, especially in the gastrointestinal tract. Upregulated Foxp3 and IL-10 mRNA expression probably indicates the induction of Tregs during the later stages of F4⁺ ETEC infection (Käser et al., 2008). Since ETEC infection causes inflammation and intestinal damage in piglets, the induction of Tregs is probably required to limit these responses and to avoid immunopathology due to an overwhelming Th17 immunity (Couper et al., 2008; Nyachoti et al., 2012). It is worth noting that F4⁺ ETEC infection triggered a significant increase in AID mRNA expression in PBMCs, Peyer's patches and MLN. AID is a B cell specific enzyme required for the class switch recombination (CSR) in activated B cells (Delker et

al., 2009). In the F4⁺ ETEC infection model, F4-specific IgG and IgA antibody-secreting cells were observed in most tissues 4 days post infection (Van den et al., 1999b; Verdonck et al., 2002). Hence, the increased AID mRNA level probably reflects ongoing class switching in B cells. Moreover, the strong induction of AID mRNA expression further supports the involvement of Th17 cells, since these cells also participate in B cell differentiation and subsequent SIgA production (Cao et al., 2012; Hirota et al., 2013; Barbosa et al., 2011; Jaffar et al., 2009).

Previous studies in our lab indicated the strong oral immunogenicity of F4 fimbriae (Van den et al., 1999a,b; Verdonck et al., 2002, 2008). To establish whether oral immunization with F4 fimbriae could elicit similar responses as an F4⁺ ETEC infection, we analyzed the cytokine mRNA expressions in PBMCs. Similar to infection, we observed a robust expression of the Th17-related genes ROR γ t, IL-23p19, IL-17F, IL-21 and IL-22. Likewise, Foxp3 and AID mRNA levels were also increased from day 4 onwards. These results indicate the capacity of F4 fimbriae to elicit Th17 responses and induce class switching in B cells upon oral administration. Unexpectedly, we failed to detect IL-17A mRNA expression. Upon ETEC infection, we observed a peak expression of IL-17A mRNA at 2 days post infection and the inability to detect IL-17A could be attributed to the later sampling point in the immunization experiment or indicate an important role for other molecules such as the enterotoxins in the IL-17A mRNA expression during infection (Loos et al., 2012). The mRNA profile in the PBMCs upon oral immunization were F4 fimbriae specific, since PBMCs from naive piglets showed an upregulated mRNA expression of Th17-related genes and an enhanced secretion of IL-17A and IL-22 upon stimulation with these fimbriae. On top of that, these responses were further amplified in the PBMCs isolated from F4⁺ ETEC infected animals upon F4 fimbriae stimulation. In contrast, all the Th1- (except IFN- γ) and Th2-related genes did not show any significant change in naive PBMCs in the first 72 h, which corroborates a previous study (Verfaillie et al., 2001). In addition, Th1 effector cells and Tregs appear to be less important during F4⁺ ETEC infection, since no significant antigen specific IFN- γ and IL-10 recall responses were obtained by restimulation with F4 fimbriae.

In conclusion, F4⁺ ETEC infection and oral immunization with F4 fimbriae elicited robust expression of Th17-related genes and IL-17 producing T cells, indicating that Th17 effector cells participate in the protective immunity to ETEC infection in piglets and that these Th17 responses are in part induced by F4 fimbriae. Moreover, we also hinted at the potential participation of IL-17B and IL-17F in the clearance of F4⁺ ETEC infection. Altogether, our results could facilitate the design of ETEC vaccines.

Chapter 5

Enterocyte and goblet cell-derived IL-17A regulates epithelial barrier fortification upon enterotoxigenic *Escherichia coli* infection

Luo Y, Loos M, Devriendt B, Cox E. Small intestinal epithelial cells respond to ETEC infection by IL-17A regulated barrier fortification (Submitted).

Author Contributions

YL: study design, experiments, and first draft of the manuscript; ML: generated isogenic ETEC mutant strains, performed the SISP experiments; YL, BD, and EC: study design and experiments, data analysis and interpretation. BD and EC revised the manuscript.

Abstract

Small intestinal epithelial cells (IECs) are the first cells encountering intestinal pathogens. These cells recognize pathogens and relay this information to innate immune cells, which then secrete IL-17A and IL-22. These cytokines play a major role in the fortification of the intestinal epithelial barrier. Here, we provide evidence for an additional protective layer as we report the previously uncharacterized ability of small IECs to produce IL-17A in response to early and late infection with enterotoxigenic *E. coli* (ETEC). The ETEC-specific enterotoxin STb was sufficient to trigger IL-17A by small IECs, goblet cells and enterocytes. This epithelial-derived IL-17A increased the production of IL-17C, mucins, antimicrobial peptides and plgR by small intestinal epithelial cells, further highlighting the role of IL-17A in epithelial barrier fortification upon bacterial infection. Our findings provide novel insights in the function of the intestinal epithelium during infection and may inform on the development of therapeutics aiming at enhancing the barrier function of the intestinal epithelium.

5.1 Introduction

The gastro-intestinal surfaces constitute the largest surface area of the body and are continuously exposed to a vast array of foreign and potentially hazardous molecules. These tissues are protected from potential insults by a single cell layer, mostly composed of absorptive enterocytes and goblet cells, which form a physical barrier (mucus, glycocalyx, antimicrobial proteins) to keep both commensals and pathogens at bay (Peterson and Artis, 2014). However, enterocytes are also able to respond to pathogens, such as enterotoxigenic *E. coli* (ETEC), resulting in the production of pro-inflammatory chemokines and cytokines, such as CCL20, IL-8 and IL-6, to alert neighboring epithelial cells and underlying immune cells of an ongoing infection (Devriendt et al., 2010b; Zanella et al., 2011a,b; Zhou et al., 2012). These immune cells then secrete IL-17A, IL-17F and IL-22, which trigger increased production of tight junction proteins, antimicrobial peptides (AMPs) and cytokines by epithelial cells, resulting in barrier fortification (Cua and Tato et al., 2010; Lee et al., 2015; Maxwell et al., 2015). Moreover, recent data indicate that IL-17A plays an important role in the onset of inflammatory bowel disease (IBD) as it amplifies the production of IL-17C by goblet cells and enteroendocrine cells and as such regulates the epithelial barrier function (Reynolds et al., 2012; Friedrich et al., 2015).

ETEC infections are still a major health burden to man and livestock species, causing severe morbidity and mortality. Especially young animals, children in and travellers to areas where ETEC strains are endemic are affected (Kotloff et al., 2013). In pigs, ETEC infections cause neonatal and postweaning diarrhea, leading to severe economic losses in swine husbandry worldwide (Fairbrother et al., 2005). ETEC possess several virulence factors governing its pathogenesis. These bacteria produce fimbriae on their surface enabling them to adhere to fimbriae-specific receptors in the apical membrane of the small intestinal epithelium. In addition, these pathogens secrete enterotoxins – heat-labile enterotoxin LT and heat-stable enterotoxins STa/STb – which disrupt the water and electrolyte balance in the gut. Upon secretion, LT and STa enterotoxins trigger the host cell production of cyclic nucleotides (cAMP and cGMP, respectively), which activate protein kinases that ultimately results in the activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and a

Na⁺ ion exchanger on the apical membrane of intestinal epithelial cells (IECs). The subsequent chloride secretion through the CFTR channel as well as the loss of salt and water into the intestinal lumen leads to the watery diarrhea characteristic of ETEC infections (Fleckenstein et al., 2010; Sheikh et al., 2014). STb on the other hand binds to sulfatide, resulting in Ca²⁺ mobilization and Cl⁻ secretion via the activation of CFTR. In addition, STb triggers the synthesis of prostaglandin E₂ (PGE₂) and 5-hydroxytryptamine (5-HT), which mediate secretion of H₂O and HCO₃⁻ and prevent Na⁺ absorption by enterocytes, leading to diarrhea (Dubreuil, 2008; Nassour and Dubreuil, 2014).

Previously, we found that the ETEC-specific STb enterotoxin plays an important role in the induction of diarrhea early during infection. This coincided with an STb-specific innate immune response by the host, which was characterized by elevated IL-1 β and IL-17A transcripts in the small intestinal tissue 4h upon infection (Loos et al., 2012). IL-17A is mainly known as the hallmark cytokine of Th17 cells, however, the kinetics of this response exclude Th17 cells as the main source (Angkasekwinai and Dong, 2011). IL-17A can, however, also be produced by innate immune cells, including innate lymphoid cells (ILC) 3, $\gamma\delta$ T cells, invariant natural killer (iNKT) cells, lymphoid tissue inducer (LTi) cells, neutrophils and macrophages and even Paneth cells (Di Padova et al., 2013). Despite the emerging protective role of innate IL-17A in gut immunity due to its regulation of the epithelial barrier function, few studies have pinpointed its cellular source during infection. Here, we aimed to identify the cellular source of these innate IL-17A transcripts upon ETEC infection.

5.2 Material and methods

The methodology of the animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2014/01).

5.2.1 Bacterial strains and culture supernatants

ETEC strains (**Table 1**) were cultured as previously described (Loos et al., 2012). Briefly, the frozen bacteria were first recovered on the Brain Heart Infusion (BHI; Oxoid, Hampshire, UK) agar plate and then bacterial colonies of each strain were transferred

into Casamino Acids-Yeast Extract (CAYE) medium pH 8.2, and grown overnight at 37°C with shaking. OD values at 650 nm of all cultures from different strains were adjusted to the same value with CAYE medium. Then, the corrected culture supernatants were spun down and filtered through 0.22 µm low protein-binding filter (Millipore, Massachusetts, USA). Filtered bacterial culture supernatant were stored at -80 °C for future use.

Table 1 Bacterial strains used in this study

Strain	Description	Toxin phenotype	Reference
HB101	Non-pathogenic <i>E. coli</i> strain		
GIS26 wild type	Wild-type F4+ ETEC reference strain	STa ⁺ STb ⁺ LT ⁺	
GIS26ΔfaeG	F4 fimbriae deficient mutant of GIS26 wild type	STa ⁺ STb ⁺ LT ⁺	Verdonck et al., 2008
GIS26ΔeltAB	Mutant strain only expressing STb	STa ⁺ STb ⁺ LT ⁻	Loos et al., 2012
GIS26ΔestBΔeltAB	Mutant strain only expressing STa	STa ⁺ STb ⁻ LT ⁻	Loos et al., 2012
GIS26ΔestB	Mutant strain expressing STa and LT	STa ⁺ STb ⁻ LT ⁺	Loos et al., 2012
GIS26 ΔestAΔestB:KAN	Mutant strain without enterotoxin production	STa ⁺ STb ⁻ LT ⁻	Loos et al., 2012

5.2.2 Fluorescent in situ hybridization

Porcine jejunal tissue was obtained from piglets undergoing in vivo small intestinal segment perfusion (SISP) as previously described (Loos et al., 2012). Briefly, five small intestinal segments from mid-jejunum were randomly perfused with 2.5×10⁹ CFU of the ETEC reference strain GIS26 (STa⁺STb⁺LT⁺) or the isogenic mutant strains GIS26 (STa⁺STb⁺LT⁻), GIS26 (STa⁺STb⁻LT⁻) and GIS26 (STa⁻STb⁻LT⁻) or PBS (**Table 1**). Four hours after perfusion, a small piece of tissue of each segment was sampled and embedded in methocel (Sigma-Aldrich, Diegem, Belgium), snapfrozen in liquid nitrogen and stored at -80°C until cryosectioning. To detect IL-17A mRNA in the methocel-embedded tissues, the QuantiGene ViewRNA ISH Tissue Assay Kit (Affymetrix, Santa Clara, CA, USA) was used according to the guidelines with minor modifications. Briefly, the samples were sectioned at 12 µm and mounted onto Fisherbrand Superfrost Plus (white label) microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany). Then, the slides were immediately fixed in chilled 4%

formaldehyde for 16-18 hours at 4°C. The next day, the slides were washed 3 times with sterile PBS and dehydrated with a graded ethanol series (50%, 70% and 100%). After drying for 30 min at 60°C, the sections were digested in protease solution for 30 min at 40°C. The protease digestion time was optimized beforehand. Subsequently, the sections were washed 3 times with sterile PBS and then submerged into 4% formaldehyde for 5 min at room temperature (RT). The sections were then hybridized with porcine IL-17A probe sets or β -actin probe sets (provided in the kit) as control at 40°C for 3 h. The targeted signal was further amplified by PreAmplifier and Amplifier (provided in the kit) hybridization at 40°C for 25 min and 15 min respectively. The sections were washed 3 times in wash buffer (provided in the kit), followed by Label Probe-AP hybridization at 40°C for 15 min. After 3 washes with wash buffer, color reactions were carried out by applying the Fast Red Substrate at 40°C for 30 min. Nuclei were counterstained with Hoechst (10 μ g/mL, Sigma-Aldrich) for 15 min at RT. Finally, slides were washed with ultrapure water and mounted in glycerol containing 0.223 M 1,4-diazobicyclo-(2,2,2)-octane (Sigma-Aldrich) and imaged on a confocal microscope (Leica TCS SP2, Leica Microsystems GmbH).

5.2.3 Immunohistochemistry

ETEC infection was performed as previously described in the last chapter (see also Luo et al., 2015). Briefly, piglets were inoculated with the ETEC reference strain GIS26 (O149:K91:F4ac⁺, LT⁺STa⁺STb⁺, 10¹⁰ CFU in 10 mL sterile PBS) or PBS on day 0 (D0) and day 1 (D1). On D4, piglets were euthanized and ileal tissue was collected, embedded in 2% (w/v) methocel, snapfrozen in liquid nitrogen, and stored at -80 °C.

The cryosectioning and staining procedures were performed as previously described in the last chapter (see also Luo et al., 2015). The primary antibodies used include anti-CD3 (5 μ g/mL, rat IgG₁, AbD Serotec, Oxford, UK), anti-CD172a (undiluted hybridoma supernatant, clone 74-22-15, mouse IgG₁), anti-cytokeratin peptide-18 mAb (CK18, mouse IgG₁, Sigma-Aldrich, Bornem, Belgium, 1:100), anti-MUC2 mAb (0.5 μ g/mL, mouse IgG₁, clone Ccp 58, Thermo Fisher Scientific, Carlsbad, CA, USA) and anti-MUC2 pAb (0.2 μ g/mL, rabbit pAb, IgG, sc-15334, Santa

Cruz Biotechnology Santa Cruz, CA, USA), biotinylated anti-swine IL-17A (2.5 µg/mL, rabbit pAb, Kingfisher biotech, St. Paul, MN, USA), anti-IL-17C pAb (2 µg/mL, rabbit pAb, ab153896, Abcam, Cambridge, UK) and anti-secretory component (SC) mAb (undiluted hybridoma supernatant, clone K60 1F1, mouse IgG₁). The next day, sections were incubated with the secondary antibody in different combination including Texas Red-X conjugated goat anti-mouse IgG (H+L) (10 µg/mL, Thermo Fisher Scientific, Rockford, IL, USA), FITC conjugated Goat anti-rabbit IgG (H+L) (10 µg/mL, Thermo Fisher Scientific), Alexa Fluor 594 conjugated goat anti-rat IgG (H+L) (10 µg/mL, Thermo Fisher Scientific), FITC-streptavidin (2.5 µg/mL, Biolegend, London, UK) and Alexa Fluor 647 conjugated goat anti-mouse IgG (H+L) (10 µg/mL, Thermo Fisher Scientific). The nuclei were counterstained with Hoechst (10 µg/mL) and the slides were mounted in glycerol containing 0.223 M 1,4-diazobicyclo-(2,2,2)-octane (Sigma-Aldrich) and imaged on a confocal microscope.

5.2.4 Cell culture and bacterial inoculum

The porcine intestinal epithelial cell line IPEC-J2, isolated from the jejunum of a neonatal piglet (Berschneider, 1989), was grown in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12 1:1; ThermoFisher Scientific) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamin (ThermoFisher Scientific), 1× Insulin/Transferrin/Selenium (ITS, Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (ThermoFisher Scientific) and 5 ng/ml human epidermal growth factor (EGF, ThermoFisher Scientific) at 37°C and 5% CO₂ in T75 cell culture flasks in a humidified atmosphere. These cells (5×10⁵ cells/well) were seeded in a 24-well cell culture plate and maintained in IPEC-J2 culture medium for 24 h. Next, the culture medium was replaced with IPEC-J2 culture medium without FCS (differentiation medium) to induce differentiation. Prior to bacterial inoculation, the differentiated cells were gently washed 3 times with sterile PBS and cultured in differentiation medium without antibiotics for 2 hours at 37°C, 5% CO₂, 90% humidity. Then the cells were inoculated with the different ETEC strains (5×10⁷ CFU/well, MOI = 100) in antibiotic-free differentiation medium and incubated at 37°C, 5% CO₂ in a humidified atmosphere. After 1 h of incubation, cells were lysed in 1 mL TRIzol immediately after

aspiration of the culture medium or further cultured in differentiation medium supplemented with 50 µg/mL gentamycin (Thermo Fisher Scientific) after 3 times washing with PBS to remove non-adherent bacteria. One hour later, IPEC-J2 cells were harvested and lysed in TRIzol or incubated for another 24 h. Subsequently, the cell supernatants were collected and stored at -80°C, while cells on the coverslips were fixed with 4% paraformaldehyde for 5 min at RT and stained with anti-swine IL-17A and anti-MUC2 as described above.

IPEC-J2 were also cultured in the presence of filtered bacterial culture supernatant (25 µL, 50 times diluted), purified STb (kindly provided by Prof. Dr. Daniel Dubreuil J, Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Québec, Canada), IL-17A (100 ng/mL). Before that, IPEC-J2 cells were first pretreated with anti-IL-17A Ab (10 µg/mL, Kingfisher biotech) or control antibody (10 µg/mL, rabbit polyclonal IgG, Abcam) for 2 h to block the IL-17A signaling. Cells were collected 2 and 24 h after SN stimulation, lysed in 1 mL TRIzol and stored at -80°C until RNA extraction. At 24 h, cells were fixed and stained with anti-MUC2, anti-IL-17C and anti-CgA as described above. Meanwhile, cell supernatant was collected and stored at -80°C until further processing.

5.2.5 Real-time qPCR

RNA was isolated using TRIzol Reagent (ThermoFisher Scientific) and purified with DNase I treatment and RNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands) following the manufacturer's instructions. RNA concentration and purity were further checked by measuring the optical density at OD260/OD280 and OD260/OD230 with a NanoDrop 2000/2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only RNA samples had OD260/OD280 ratios between 1.8 and 2.0 and OD260/OD230 ratios between 1.9 and 2.1 will be selected and reverse transcribed by SuperscriptTM III Reverse Transcriptase (200 U for 1 µg RNA; Thermo Fisher Scientific) using random primers (7.5 ng/µL for 1 µg RNA, Thermo Fisher Scientific). Primers (**Table 2**) were designed using Primer 5 or an online tool to span an exon-exon junction, thereby avoiding amplification of genomic DNA. The primers used in this study were strictly selected with amplification efficiency ranged from 95% to 105%

and all corresponding amplification products were sequenced afterwards. Quantitative PCR (qPCR) assays using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) were carried out on the StepOnePlus real-time PCR system (Applied Biosystems).

Table 2 List of the primers used in the qPCR assay.

Gene	Sequence (5'→3')	size (bp)	Ta (°C)	Reference
IL-8	F:CAAGCAAAAACCCATTCTCCG	99	58	Zhou et al., 2012
	R:CCAGCACAGGAATGAGGCATA			
TNF-α	F:ACTGCACTTCGAGGTTATCGG	118	60	Meissonnier et al., 2008
	R:GGCGACGGGCTTATCTGA			
IL-1β	F:GAGGCAGCAGCTCGGAAAAT	87	60	Devriendt et al., 2009
	R:TCCCGGGTGATGTTGTAATCC			
IL-17A	F:ACTCCAAACGCTTCACCTCAC	234	58	Luo et al., 2015
	R:AGCCCACTGTCACCATCACTT			
IL-17C	F:CGTGTGGACACGGATGAGAG	217	60	Present study
	R:GGATGAACTCGGCGTGGAAG			
IL-17RA	F:CCAGACGCTGTTGAAGACCT	230	60	Present study
	R:GCCGAGTAGACGATCCAGAC			
IL-17RB	F:GGGGGTAGAAGTGCTGACCA	291	60	Present study
	R:GTCCTTTAATGCCACAGCATTTAGT			
IL-17RC	F : CCTTCAGGCCTACCTACT	171	57	Present study
	R: GTTCTTCTGGTACCTGGGC			
IL-17RE	F:CCCAGATTCCTCGCCATACC	106	60	Present study
	R:CCTGGCAACAGATACAGGCA			
plgR	F:AGCCAACCTCACCAACTTCC	105	62	Trevisi et al., 2013
	R:CTGCTAATGCCAGACCAC			
MUC2	F:ACCCGCACTACGTCACCTTC	150	62	Bruehl et al., 2010
	R:GGCAGGACACCTGGTCATTG			
PBD-2	F:TTGCTGCTGCTGACTGTCTG	180	62	Meurens et al., 2010
	R:CTTGGCCTTGCCACTGTAAC			
HPRT1	F: ACACTGGCAAAACAATGCAA	71	60	Støy et al., 2013
	R:TGCAACCTTGACCATCTTG			
β-actin	F:TCATCACCATCGGCAACG	133	60	von der Hardt et al., 2004
	R:TTCCTGATGTCCACGTCGC			
GAPDH	F: GGGCATGAACCATGAGAAGT	230	60	Melkebeek et al., 2007
	R: AAGCAGGGATGATGTTCTGG			

5.2.6. IL-17A cytokine ELISA

The secretion of IL-17A in cell-free supernatants was measured using the commercial ELISA kit according to the manufacturer's guidelines (Swine IL-17A ELISA VetSet, Kingfisher biotech).

5.2.7. Statistical analysis

Statistical analysis was performed with the Mann-Whitney U test or Kruskal-Wallis Test for the independent samples and Friedman's Two-Way Analysis for the related samples in the SPSS 22 software package. The significance level was set at $p < 0.05$.

5.3 RESULTS

5.3.1 Early IL-17A mRNA production in small intestinal epithelial cells after ETEC infection

Our previous work demonstrated that F4⁺ ETEC infection induced early IL-17A mRNA in the jejunum (Loos et al., 2012). In order to identify the cellular source of this mRNA, fluorescent in situ hybridization (FISH) was applied to cryosections of intestinal tissues perfused for 4h with the wild type ETEC strain GIS26. As shown in **Figure 1**, IL-17A mRNA levels were upregulated in the small intestine upon infection with GIS26 as compared to controls. Surprisingly, IL-17A transcripts mainly localized within epithelial cells at the tip of the villi and in the crypts, although scattered IL-17A transcripts were also found in the subepithelial tissue of the lamina propria (**Figure 1**, asterisk). These findings imply that small IECs respond within hours to ETEC infection by upregulating IL-17A transcript levels.

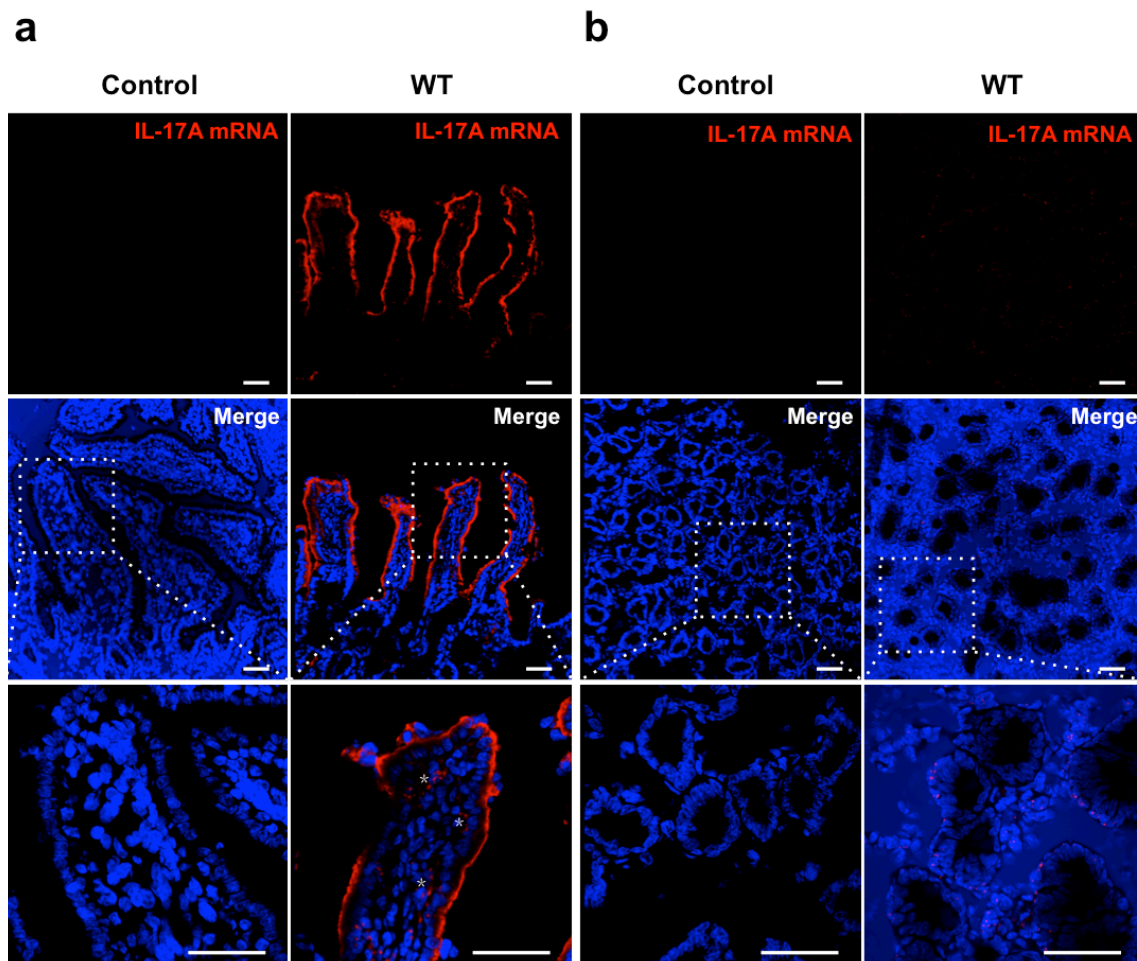


Figure 1. Small intestinal epithelial cells produce IL-17A mRNA upon bacterial infection. Piglets were inoculated with F4⁺ETEC or vehicle for 4h in the SISP model. Cryosections were hybridized with IL-17A mRNA probe sets and the nuclei were counterstained with Hoechst. Representative confocal micrographs (n = 2) from villi **(a)** or crypts **(b)** of either GIS 26 (STa⁺STb⁺LT) (**WT**) or PBS (**Control**) perfused animals. Scale bar = 50μm.

5.3.2. Enterocytes and goblet cells produce IL-17A and C upon ETEC infection

In a next effort we sought to elucidate if this epithelial IL-17A production also occurred at later stages of ETEC infection. To this end, piglets were inoculated with the ETEC WT strain GIS26 and intestinal tissues were sampled at day 4 to assess IL-17A production by different cell populations. As expected we observed an influx of CD3⁺ T cells and CD172a⁺ myeloid cells upon ETEC infection (**Figure 2a**). In addition, ETEC infection clearly triggered IL-17A production in the villi and crypts. In the latter, IL-17A is produced by CD3⁺ T cells and a CD3⁻CD172a⁻ population (**Figure 2a, downwards**

and upturned arrowheads respectively), while in the villi the majority of the IL-17A⁺ cells are CD3⁺CD172a⁻ (**Figure 2a, arrows**). In addition, the location of the IL-17A signal, along the villus and in the crypts, pointed towards epithelial cells as the main source of IL-17A. In an effort to identify which epithelial cells produced IL-17A, tissues were stained with cytokeratin 18 (CK18), a marker for porcine goblet cells and M cells (Gebert et al., 1994; Schauser et al., 2004). This revealed that CK18⁺ epithelial cells produced IL-17A upon ETEC infection (**Figure 2b**). Most of these CK18⁺IL-17⁺ epithelial cells display a morphology resembling goblet cells (**Figure 2b, arrowheads**), implying that goblet cells respond to ETEC infection with IL-17A production. Notably, scattered CK18⁺IL-17A⁺ cells were also found in the villi and crypts of control animals (**Figure 2b, Supplementary Figure S1**).

Since colonic goblet cells and enteroendocrine cells were reported to secrete IL-17C in the context of IBD and in this setting play a pivotal role in the regulation of the epithelial barrier (Friedrich et al., 2015), we assumed ETEC infection could also trigger IL-17C production by small IECs. To verify IL-17C production by goblet cells or enteroendocrine cells, we co-stained intestinal tissues for IL-17C and CK18 or chromogranin A (CgA), respectively. Similar to IL-17A, IL-17C was induced upon ETEC infection (**Figure 3a**). Only a small population of IL-17C⁺ cells stained positive for CgA (**Figure 3a, white arrowhead**), while the majority of the IL-17C⁺ producing cells were CK18⁺ (**Figure 3b**). These findings indicate that upon ETEC infection, most IL-17C was produced by goblet cells and not enteroendocrine cells. Notably, both IL-17A and IL-17C localized at the basolateral side of the goblet cells. Moreover, unlike IL-17A, which was produced by both villus and crypt goblet cells, IL-17C mainly localized in the crypts (**Figure 3c**).

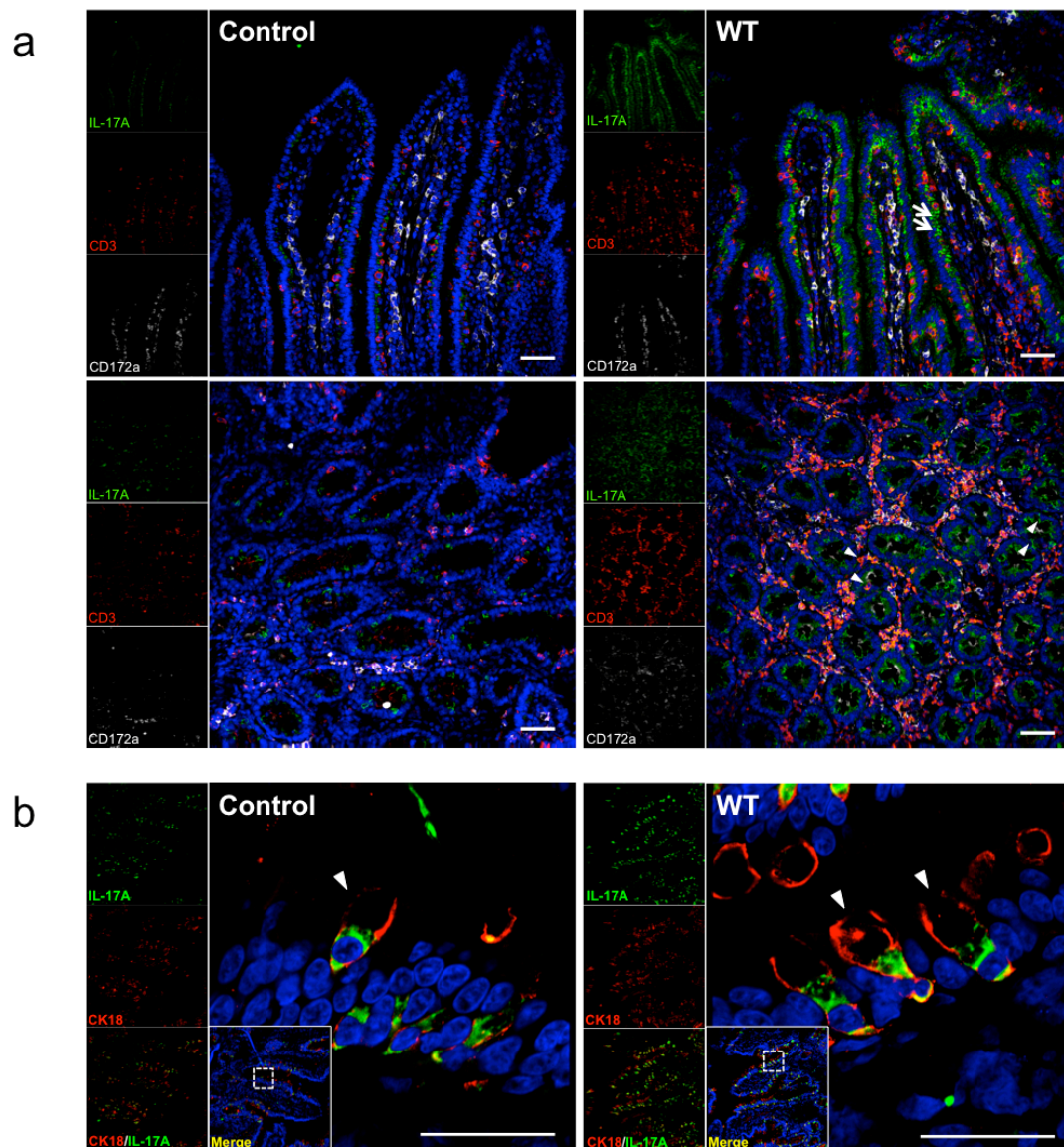


Figure 2. T cells and goblet cells produce IL-17A upon F4⁺ETEC infection of piglets. Piglets were infected with F4⁺ETEC and on day 4 intestinal tissues were sampled. Cryosections were stained with markers to identify IL-17A-producing cells. (a) Cryosections were stained with anti-IL-17A (FITC, green), anti-CD3 mAb (AlexaFluor 594, red) and anti-CD172a mAb (AlexaFluor 647, grey). (b) Cryosections were stained with anti-IL-17A (FITC, green) and anti-CK18 mAb (Texas Red-X, red). The nuclei were counterstained with Hoechst. IL-17A⁺CK18⁺ cells with goblet cell morphology are indicated with arrowheads. Representative confocal images are shown for villi and crypts of all piglets in both groups (n = 3). Scale bar = 50 μ m.

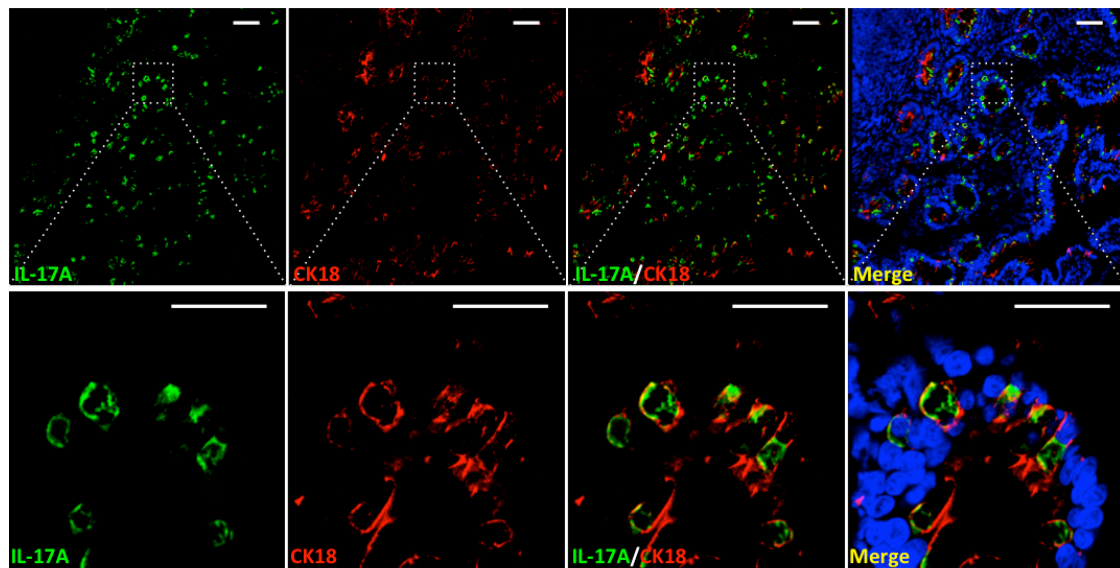


Figure S1. IL-17A staining in crypts of F4⁺ETEC infected piglets. Piglets were infected with F4⁺ETEC and on day 4 intestinal tissues were sampled. Cryosections were stained with markers to identify IL-17A-producing cells. Cryosections were stained with anti-IL-17A (FITC, green) and anti-CK18 mAb (Texas Red-X, red). The nuclei were counterstained with Hoechst. Representative confocal images are shown (n = 3). Scale bar = 50 μ m.

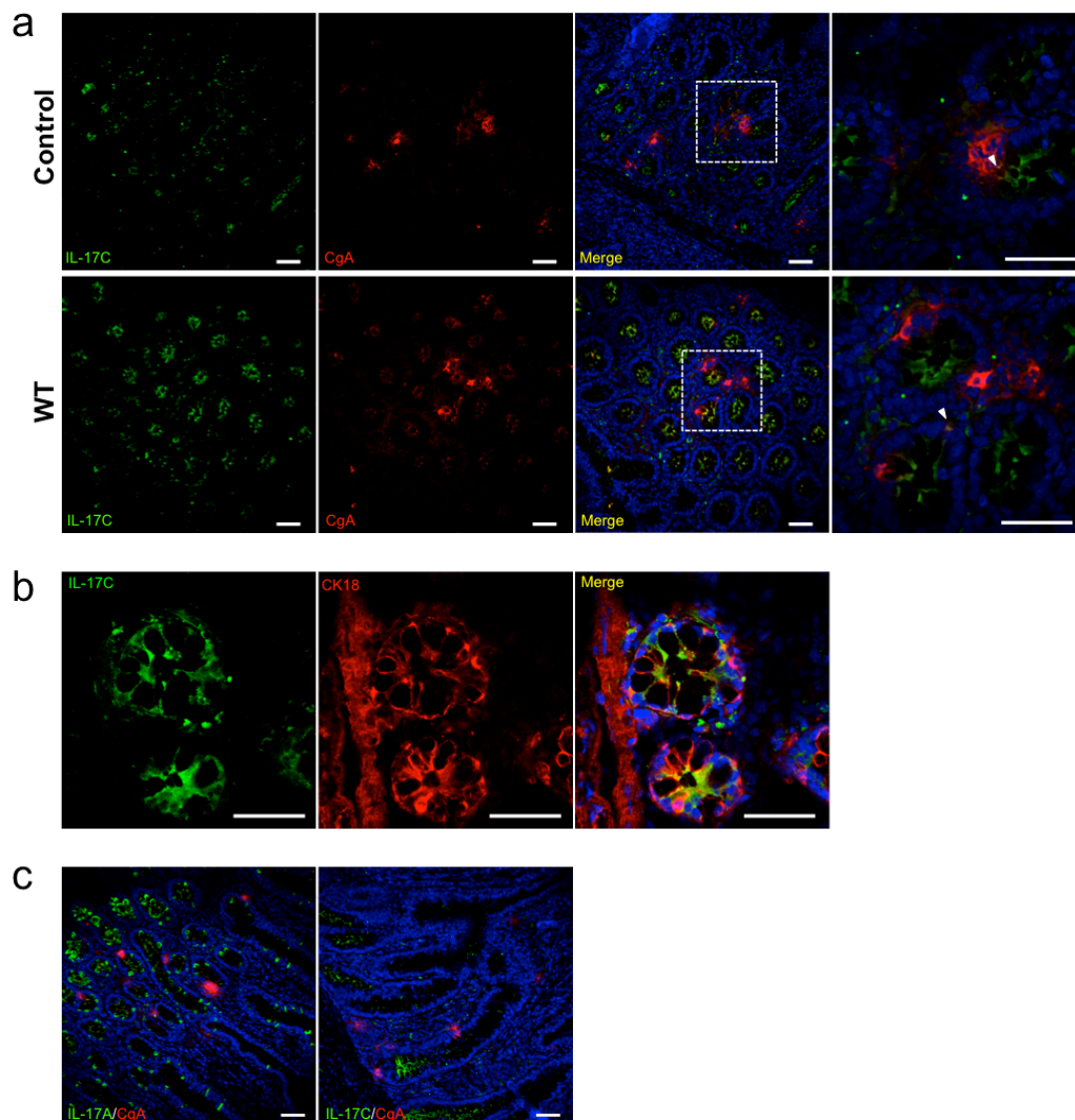


Figure 3. Goblet cells are the main source of IL-17C in F4⁺ ETEC infected piglets. Piglets were infected with F4⁺ ETEC and on day 4 intestinal tissues were sampled. Cryosections were stained with markers to identify IL-17C-producing cells. (a) Cryosections were stained with anti-IL-17C (FITC, green) and anti-CgA mAb (Texas Red-X, red), (b) with anti-IL-17C (FITC, green) and anti-CK18 mAb (Texas Red-X, red) or (c) with anti-IL-17A (FITC, green) and anti-CgA mAb (Texas Red-X, red). The nuclei were counterstained with Hoechst. Representative confocal images are shown for villi and crypts of all piglets in both groups. Scale bar = 50 μm.

5.3.3. The enterotoxin STb triggers early IL-17A mRNA production in intestinal epithelial cells

We previously demonstrated that the ETEC-specific enterotoxin STb is sufficient to trigger intestinal IL-17A mRNA production early during ETEC infection (Loos et al., 2012). To confirm the role of the STb enterotoxin in the production of IL-17A transcripts by intestinal epithelial cells, we again applied FISH on intestinal tissues obtained from piglets perfused with different enterotoxin-deficient ETEC mutants. As shown in **Figure 4**, IL-17A transcripts were detected in the jejunum after GIS26(STa⁻STb⁺LT⁻) perfusion to a similar extent as the WT strain. These IL-17A transcripts mainly localized in epithelial cells along the villus. In contrast, the mutant strains GIS26(STa⁺STb⁻LT⁻) and GIS26(STa⁻STb⁻LT⁻), which lack the STb enterotoxin, failed to induce IL-17A mRNA production (**Figure 4**). These results indicate that the presence of the enterotoxin STb *in vivo* is sufficient to drive production of IL-17A transcripts by small IECs.

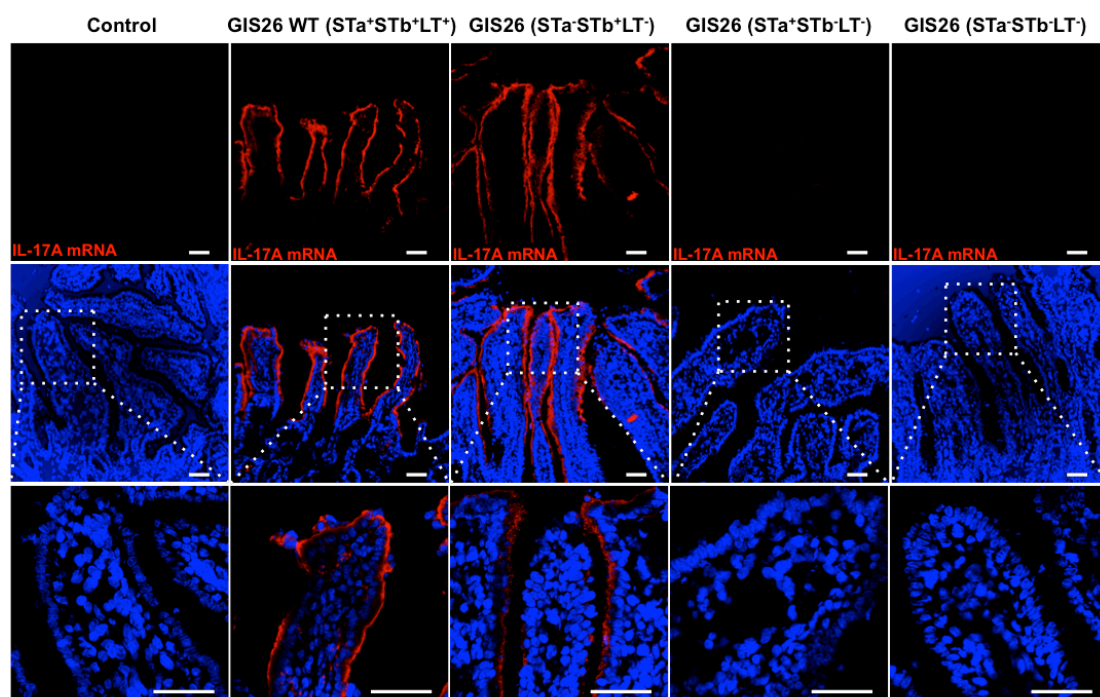


Figure 4. The STb⁺ ETEC mutant strain triggers IL-17A mRNA expression by small intestinal epithelial cells. Piglets were inoculated with the different ETEC strains or vehicle for 4h in the SISF model. Cryosections were hybridized with IL-17A mRNA probe sets and the nuclei were counterstained with Hoechst. Representative confocal images are shown (n = 2). Scale bar = 50 μ m.

5.3.4. The enterotoxin STb triggers IL-17A and IL-17C production by IPEC-J2 cells

To further confirm STb can upregulate IL-17A production by small IECs, we turned to an *in vitro* system using the non-transformed IEC cell line IPEC-J2. Monolayers were inoculated with the WT ETEC strain GIS26 and its isogenic mutant strains lacking the ability to produce one or more virulence factors as indicated in **Table 1**. Upon inoculation with the WT strain, IPEC-J2 monolayers upregulated IL-8, TNF- α , and IL-1 β transcripts as compared to the controls (**Figure 5a**), indicating that the cells properly responded to the bacteria. In addition, we further confirmed the necessity of adhesion to trigger robust pro-inflammatory responses. As expected, the mRNA expression profile upregulated by the enterotoxin-deficient strain resembled this of the laboratory *E. coli* strain (HB101), while the profile of the mutant strain expressing only STb was nearly this of the WT strain. Next, we investigated the role of the different enterotoxins in the production of IL-17 transcripts by IPEC-J2 monolayers. As shown in **Figure 5a**, WT bacteria triggered IL-17A and IL-17C mRNA expression by IPEC-J2 monolayers as compared to the laboratory *E. coli* strain HB101 and the triple enterotoxin mutant strain. Analysis of the ability of the different enterotoxin mutant strains to induce IL-17 transcripts showed that the presence of the enterotoxin STb is sufficient to trigger IL-17A and C mRNA production by IPEC-J2 monolayers (**Figure 5a**). In addition, IPEC-J2 cells responded very fast to infection, as IL-8 and IL-1 β transcript levels peaked at 1 h upon inoculation. IL-17A transcripts on the other hand were upregulated 1h after infection and were even higher at 2h, while the IL-17C mRNA response came later (**Supplementary Figure S2**).

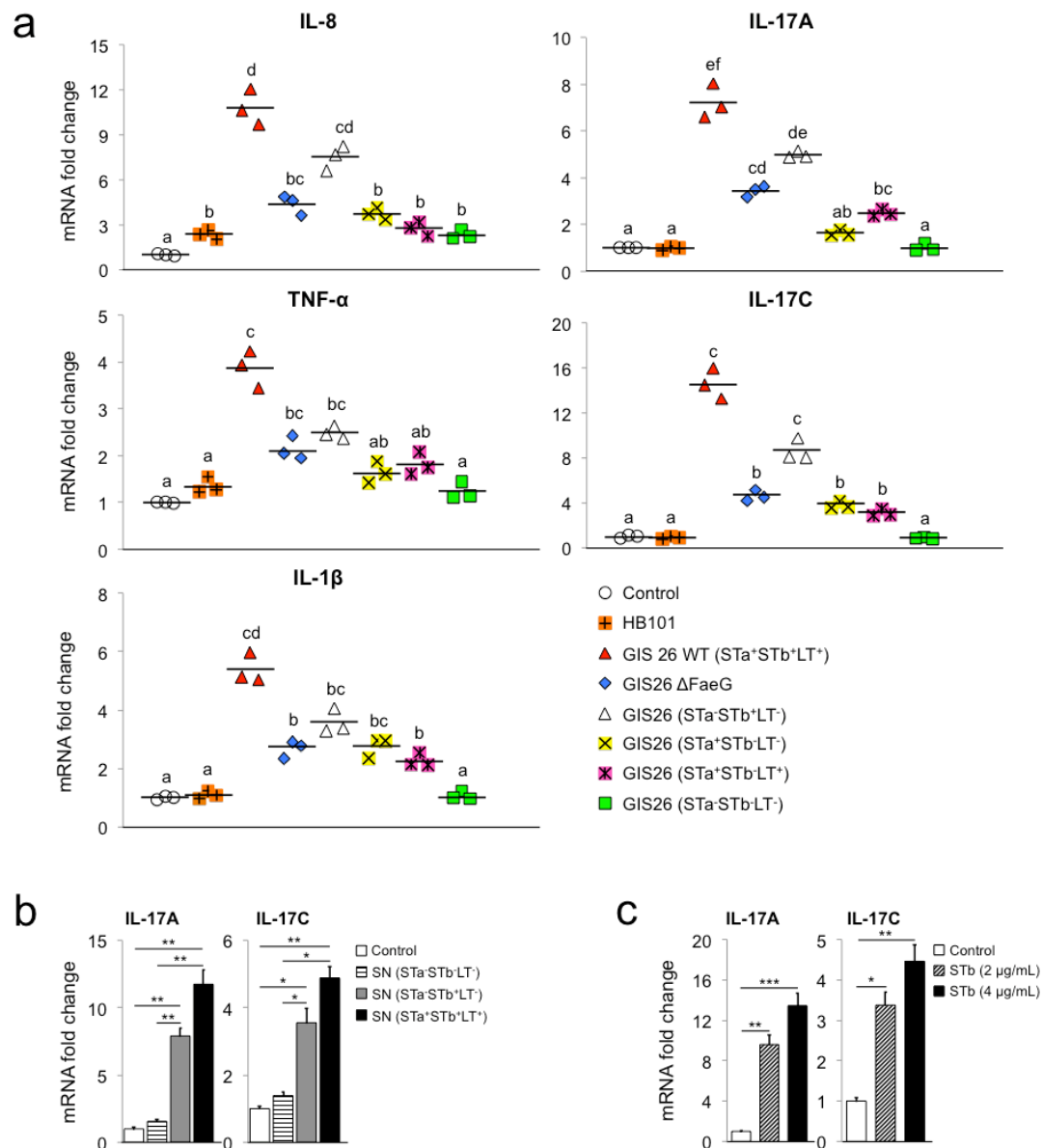


Figure 5 IPEC-J2 cells produce IL-17 cytokines upon stimulation with the STb enterotoxin. (a) IPEC-J2 monolayers were inoculated with the different bacterial strains at MOI 100 for 1h. Then, the monolayers were washed and further incubated for 1h. The mRNA expression level of the IPEC-J2 monolayers was assessed by qPCR. Different letters indicate significant differences. **(b)** IPEC-J2 monolayers were stimulated with the culture supernatants (50x diluted in medium) of the different bacterial strains or only medium as control for 2h. IL-17A and C mRNA expression by the IPEC-J2 cells was assessed by qPCR. **(c)** IPEC-J2 monolayers were stimulated with only medium as control or purified STb with different concentrations (2 and 4 μg/mL) for 2 h and IL-17A and IL-17C mRNA expression was assessed by qPCR. Data are presented as the mean ± SD obtained from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

We further confirmed these results by stimulating the IPEC-J2 monolayers with culture SN of the different ETEC mutant strains. As compared to SN of the triple enterotoxin deficient strain (STa⁻STb⁻LT⁻), SN of the WT strain and the STb⁺ mutant strain could also trigger IL-17A and C mRNA production by IPEC-J2 monolayers (**Figure 5b**). To rule out any effect of other secreted components on the IL-17 response, IPEC-J2 monolayers were stimulated with purified STb. As expected, purified STb increased IL-17A and C transcript levels by IPEC-J2 monolayers in a dose-dependent manner (**Figure 5c**). These findings demonstrate that the enterotoxin STb triggers IL-17A and C transcripts by IPEC-J2 monolayers. However, an additive effect of the enterotoxins LT and/or STa on the IL-17 mRNA production was also observed (**Supplementary Figure S3**). Moreover, we also found STb can significantly induce CCL20 mRNA expression in IPEC-J2 cells. Moreover, transcriptional levels of CCL20 are largely augmented, which seems to mediate by STa but not LT in the presence of STb (**Supplementary Figure S3**).

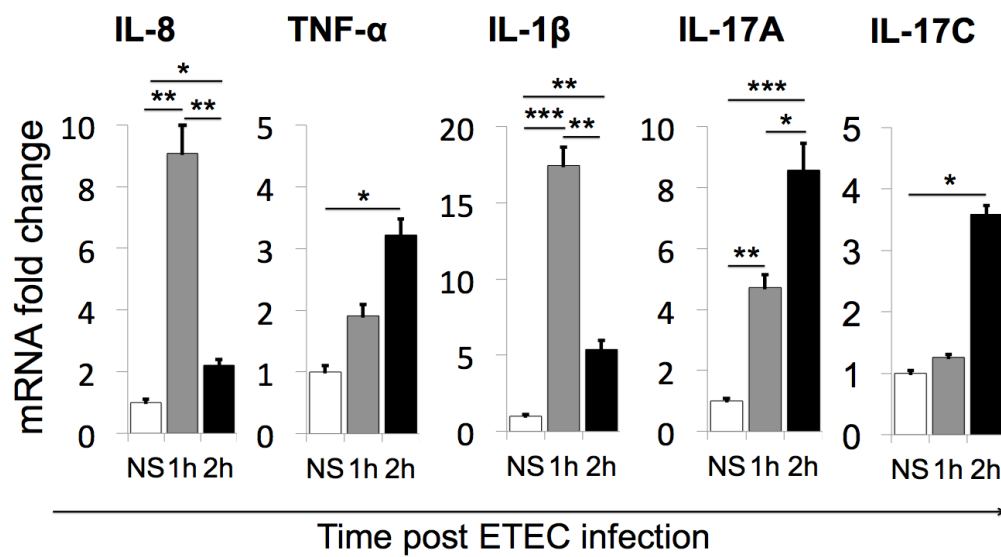


Figure S2. Cytokine mRNA expression upon F4⁺ ETEC infection of IPEC-J2 cells. IPEC-J2 monolayers were inoculated with the different bacterial strains at MOI 100 for 1 h. After that, the monolayers were directly collected for RNA extraction or washed and further incubated for another 1h. Then, the mRNA expression level of the IPEC-J2 monolayers was assessed by qPCR. Data are presented as the mean \pm SD obtained from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

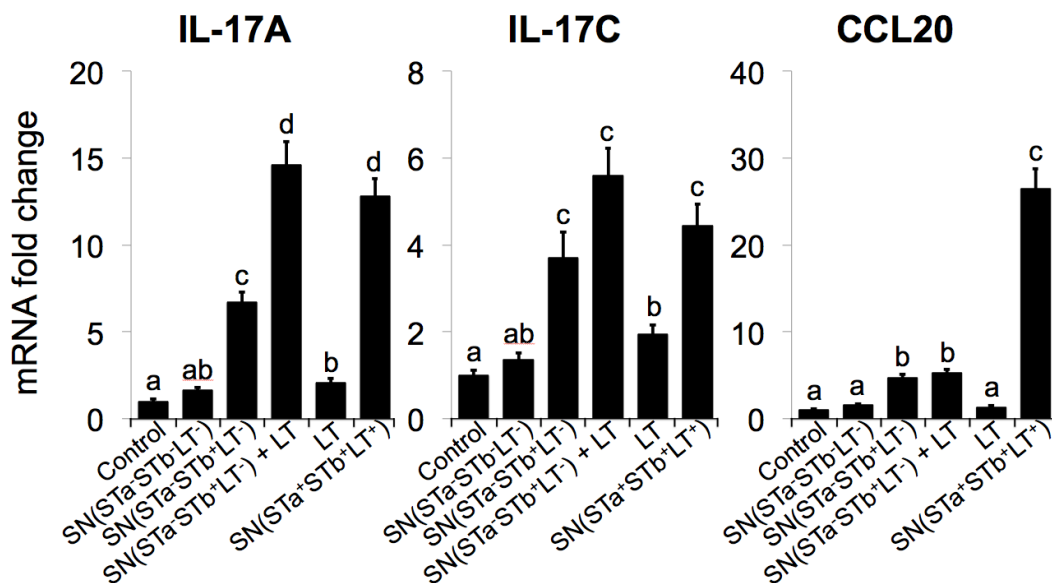


Figure S3. Effect of different enterotoxins on cytokine mRNA expression in IPEC-J2 cells. IPEC-J2 monolayers were stimulated with the culture supernatants (50x diluted in medium) of the different bacterial strains in the presence or absence of LT (2ng/mL) or only medium as control for 2h. IL-17A, IL-17C and CCL20 mRNA expression by the IPEC-J2 cells was assessed by qPCR. Data are presented as the mean \pm sd obtained from three independent experiments. Different letters indicate significant differences.

To correlate the increased IL-17A transcript levels in IPEC-J2 monolayers elicited by the enterotoxin STb with secreted IL-17A, we assessed the IL-17A level in the culture SN of stimulated monolayers by ELISA. Unfortunately, we could not detect IL-17A secretion by ETEC infected monolayers nor upon stimulation with bacterial SN or purified STb (data not shown). As IECs respond to IL-17A and have been shown to express IL-17 receptors (Lee et al., 2015; Maxwell et al., 2015; Kinugasa et al., 2000), we hypothesized that any secreted IL-17A should be immediately bound by its receptor on neighboring cells. To test this hypothesis we first assessed the expression of IL-17 receptors by IPEC-J2 cells. The IL-17A receptor is composed of a heterodimer of IL-17RA and IL-17RC, while IL-17RB/RD/RE bind other IL-17 family members (Gaffen et al., 2009). As shown in **Figure 6a**, IPEC-J2 cells express transcripts of IL-17 receptors, including IL-17RA, IL-17RB, IL-17RC and IL-17RE. Remarkably, IPEC-J2 monolayers have high constitutive IL-17RA mRNA levels (**Figure 6a**) and drastically increased IL-17RB and IL-17RE mRNA levels upon stimulation with bacterial culture SN (**Figure 6b**). The elevation in IL-17RE mRNA expression corresponds with the increased IL-17C mRNA expression, since IL-17RE specifically binds IL-17C. Moreover, we detected IL-17A and C on the surface of the IPEC-J2 cells upon inoculation with the WT strain and the GIS26 (STa⁻STb⁺LT⁻) strain as compared to controls, further supporting our hypothesis that IL-17A secreted by epithelial is immediately binding to its receptors (**Figure 6c,d**).

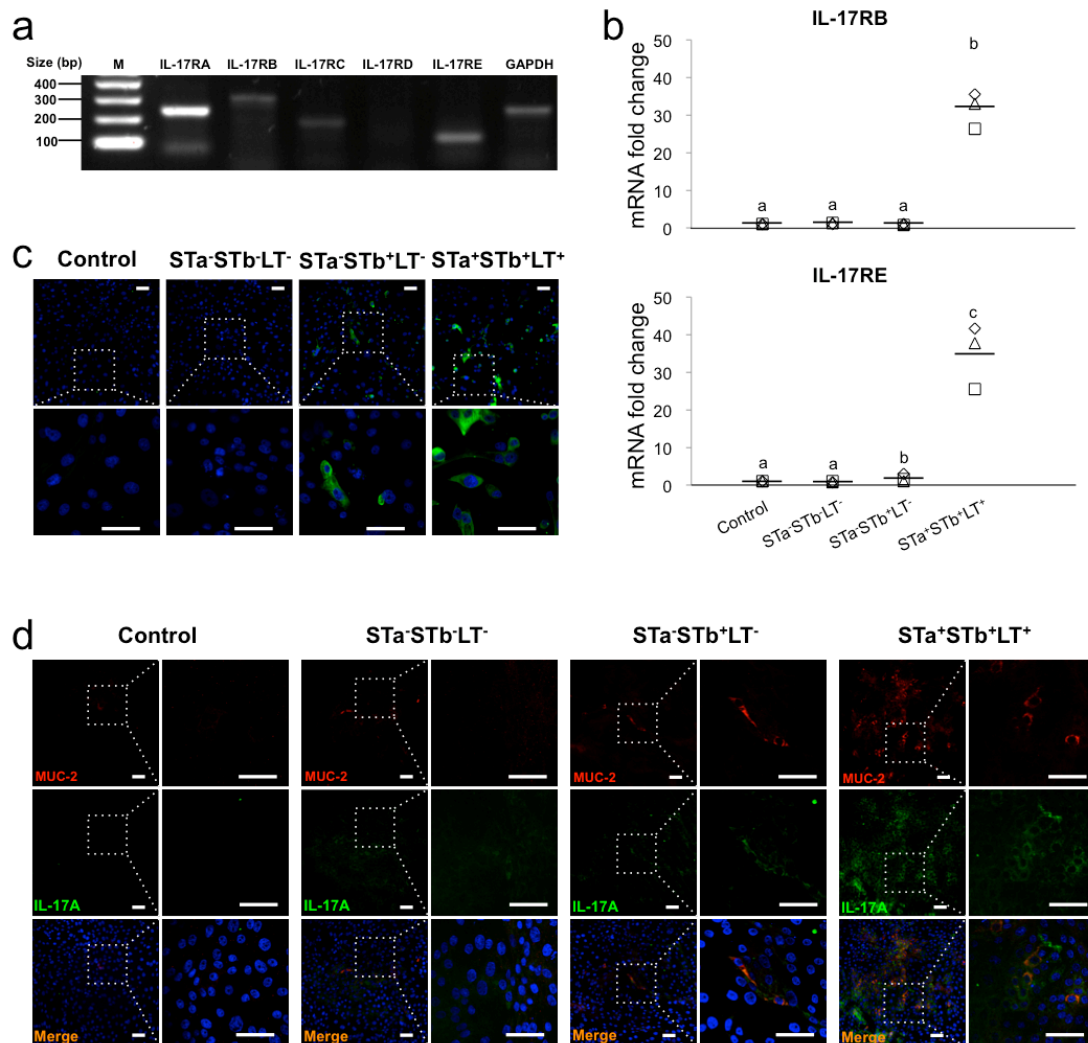


Figure 6. STb triggers IL-17 responses in IPEC-J2 cells. (a) IPEC-J2 cells express IL-17 receptor mRNA in homeostatic conditions. (b) IPEC-J2 monolayers were stimulated with culture supernatant of the different ETEC strains for 24h and the mRNA expression level of IL-17RB and IL-17RE was analyzed by qPCR. Data are presented as the mean obtained from three independent experiments. Different letters indicate significant differences. IPEC-J2 monolayers were inoculated with the different bacterial strains at MOI 100 for 1h, and then the monolayers were washed and further incubated for 24 h. Then, the cells were stained with anti-IL-17C (FITC, green) (c) or (d) anti-IL-17A (FITC, green) and anti-MUC2 antibodies (Texas Red-X, Red). The nuclei were counterstained with Hoechst and representative confocal images are shown (n = 3). Scale bar = 50 μ m.

5.3.5. STb-induced epithelial IL-17A regulates barrier fortification

IL-17A is known to improve the barrier function of IECs by eliciting upregulation of tight junction proteins, mucin production and the secretion of AMPs (Lee et al., 2015; Maxwell et al., 2015; Kinugasa et al., 2000). Recently, IL-17C produced by colonic epithelial cells was also found to promote tight junction protein expression and regulate mucosal barrier integrity in mice and human (Reynolds et al., 2012; Friedrich et al., 2015). Here, we confirmed these results in the porcine enterocyte cell line, as IPEC-J2 monolayers responded to exogenous IL-17A by upregulating transcript levels of the polymeric immunoglobulin receptor (pIgR), porcine beta-defensin 2 (pBD-2), MUC2 and IL-17C and MUC2 and IL-17C protein levels (**Figure 7a,b**). As ETEC triggered IL-17A mRNA production by IPEC-J2 cells, we reasoned that these cells should increase the expression of MUC2, pIgR and pBD-2 in response to enterotoxin stimulation. Similar to IL-17A mRNA expression, pIgR, MUC2 and pBD-2 transcript levels were increased upon stimulation of IPEC-J2 monolayers with SN from the WT strain and the STb⁺ mutant strain (**Figure 7c**). In addition, purified STb was sufficient to elicit a higher production of pIgR, pBD-2 and MUC2 transcripts. To further determine if STb mediates the IL-17A-induced upregulation of MUC2, pBD-2 as well as pIgR expression, IPEC-J2 cells were pretreated with IL-17A blocking antibodies and then stimulated with bacterial culture SN. As shown in **Figure 7d**, blocking of IL-17A reduced the mRNA expression level of pIgR, pBD-2, MUC2 as well as IL-17C to almost basal levels. Likewise, we observed an enhanced expression of MUC2 and secretory component (SC) in the small intestine of ETEC infected piglets (**Figure 7e**). Taken together, these data suggest that small IECs respond to ETEC infection by upregulating IL-17A, which regulates epithelial expression of IL-17C, pIgR, pBD-2 and MUC2 to fortify the barrier function.

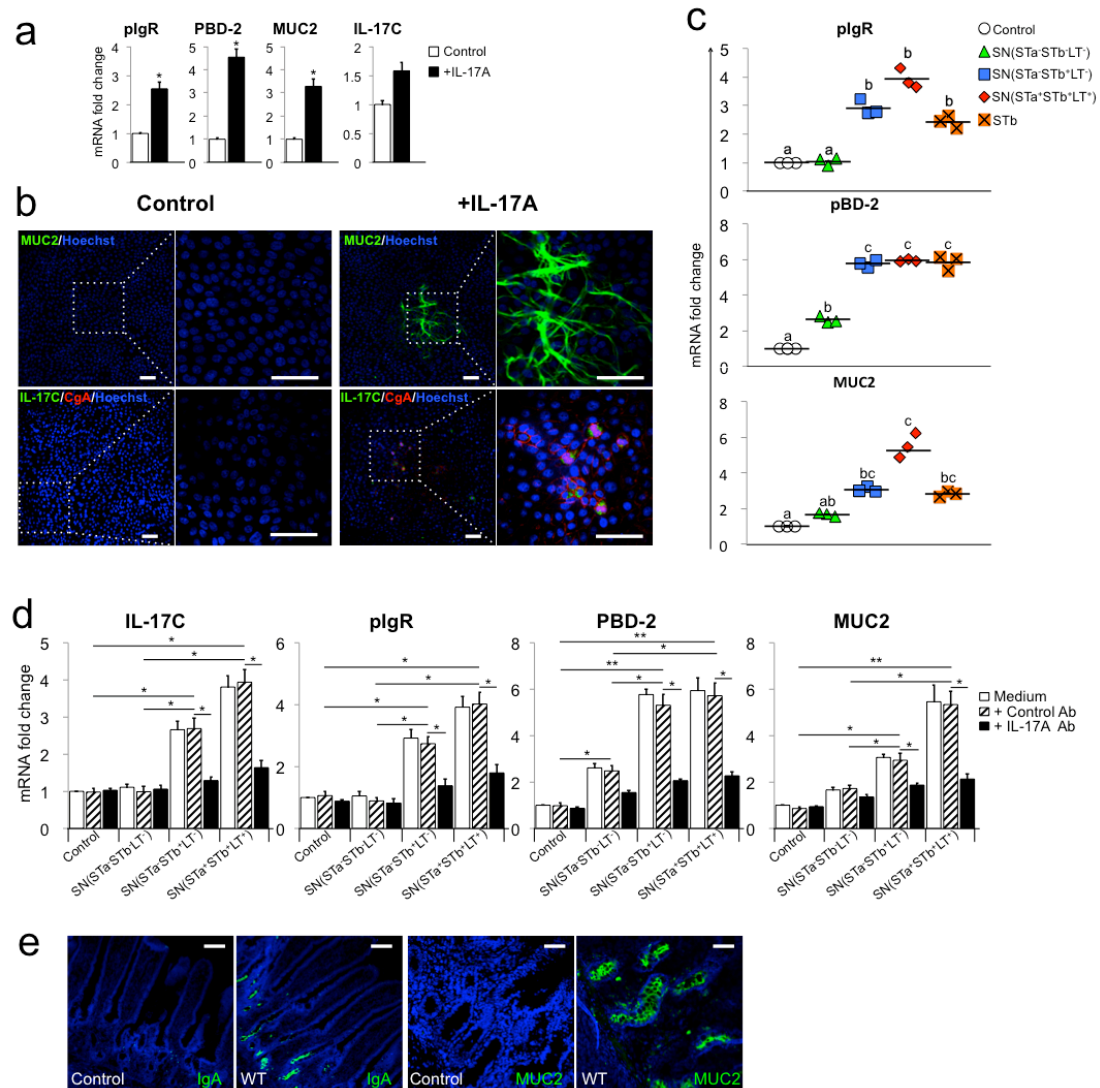


Figure 7 Role of IL-17A on the function of IPEC-J2 cells. Cells were cultured with IL-17A (100 ng/mL) or medium for 24 h. Addition of IL-17A (100 ng/mL) induced plgR, PBD-2, MUC2 and IL-17C transcripts in IPEC-J2 cells (**a**) and MUC2 and IL-17C expression (**b**). Cells were stained with anti-MUC2 (FITC, green) or anti-IL-17C (FITC, green) combined with anti-CgA (Texas Red-X, Red), the nuclei were counterstained with Hoechst (blue). Representative confocal images of three independent experiments are shown. Scale bar = 50 μ m. (**c**) IPEC-J2 monolayers were stimulated with the culture supernatans (50x diluted) of the different bacterial strains or purified STb (2 μ g/mL) for 24 h. The mRNA expression level of plgR, PBD-2 and MUC2 was analyzed by qPCR. (**d**) The presence of IL-17A pAb reduced the STb mediated mRNA expression of plgR, PBD-2, MUC2 and IL-17C in IPEC-J2 cells after stimulation with bacterial culture supernatants. IPEC-J2 cells were first treated with IL-17A pAb (10 μ g/mL) or the control Ab (rabbit IgG, 10 μ g/mL) for 2h, and then stimulated with medium in the presence or absence of bacterial culture supernatants (50x diluted) for 24h. Data are presented as the mean \pm SD obtained from at least three independent experiments. * $p < 0.05$. (**e**) The ETEC infection triggers IgA and MUC2 expression in the ileum. Four days after F4⁺ETEC infection, intestinal tissues from infected piglets (**WT**) and control piglets (**Control**) were sampled and cryosections were stained with anti-secretory component (SC) (FITC, green) or anti-MUC2 antibodies (FITC, green) and the nuclei were counterstained with Hoechst (blue). Representative confocal images of three independent experiments are shown. Scale bar = 50 μ m.

5.4 Discussion

Enterocytes are not a mere physical barrier to prevent invasion of the gastro-intestinal tissues with foreign molecules, but they recognize and respond to changes in the lumen and relay this information to underlying innate immune cells (Peterson and Artis, 2014; Devriendt et al., 2010b). These innate immune cells, such as $\gamma\delta$ T cells, secrete IL-17A, which was recently shown to regulate the epithelial barrier integrity, at least in the context of murine colitis models (Lee et al., 2015; Maxwell et al., 2015). Here, we further expand this current paradigm as we report the previously uncharacterized ability of small IECs to produce IL-17A early upon ETEC infection to fortify their barrier function by upregulating mucins, AMPs and pIgR expression. The latter is involved in the transcytosis of SIgA to the lumen to neutralize toxins and bacterial adherence. We further prove that this epithelial IL-17A-mediated barrier fortification is dependent on the recognition of the ETEC-specific enterotoxin STb by IECs.

We observed a very rapid (within 4 h) IL-17A mRNA production by small intestinal epithelial cells upon perfusing the gut with ETEC (Loos et al., 2012). Although we did not identify the epithelial source of this very early innate IL-17A mRNA after ETEC infection, goblet cells were found as the main IL-17A producing cells at a later stage of infection (day 4), a timepoint during ETEC infection at which adaptive immunity kicks in (Verdonck et al., 2002). Based on these findings and our previous work (Luo et al., 2015), we propose that early during ETEC infection, enterocytes and goblet cells produce IL-17A upon recognition of the enterotoxin STb to fortify the epithelial barrier, allowing time for innate immune cells to activate pathogen-specific adaptive immune cells, such as Th17 cells, to protect the host against infection. At later timepoints during ETEC infection, goblet cell-derived IL-17A might assist innate and adaptive immunity to regulate barrier integrity.

A recent study demonstrated STb induced the redistribution of the tight junction (TJ) protein claudin-1 to the cytoplasm in the human epithelial cell line T84, which might contribute to the pathogenesis of ETEC by allowing the passage of electrolytes and water through the increased permeability of the epithelial barrier (Nassour and Dubreuil, 2014). The increased IL-17A production by small IECs after ETEC infection

may also be involved in modulating TJ proteins, since IL-17A induces the formation of TJ complexes, promotes the transcription of claudin-1 and claudin-2 and regulates the subcellular distribution of occluding (Lee et al., 2015; Kinugasa et al., 2000). IL-17A not only regulates TJ complexes, but also triggers increased production of AMPs and MUC2. In this context it is noteworthy that ETEC are able to degrade small intestinal MUC2 in an ETEC autotransporter A (EatA)-dependent manner, thus promoting bacterial colonization to the small intestinal epithelium and accelerating toxin access to the epithelial surface (Kumar et al., 2014; Luo et al., 2014). Presumably, the IL-17A-mediated increased MUC2 production upon ETEC infection serves to replace the degraded mucus layer. Although the enterotoxin STb was sufficient to elicit IL-17A-mediated barrier fortification in IECs, we observed higher responses in the presence of LT and STa, implying that these enterotoxins might amplify the STb-mediated epithelial response. It would be interesting to pinpoint the exact contribution of LT and STa in these responses.

Similar to other models we found that porcine intestinal epithelial cells express transcripts of the IL-17 receptors, except for IL-17RD. In line with their responsiveness to IL-17A and previous observations (Ramirez-Carrozzi et al., 2011), porcine IECs have a high constitutive expression of IL-17RA. Surprisingly, IL-17RB and IL-17RE transcripts were increased after WT ETEC infection, and this seems to depend on STa and/or LT. IL-17RB binds IL-17B and IL-17E (also known as IL-25) (Gaffen, 2009). As recent data indicate that IL-17B inhibits IL-17E-mediated colonic inflammation during *Citrobacter rodentium* infection, it is tempting to speculate that the upregulated IL-17RB expression by IECs might hint at a similar mechanism during ETEC infection (Reynolds et al., 2015). IL-17RE on the other hand is the receptor for IL-17C, an epithelial specific IL-17 cytokine regulating the epithelial innate immune responses to bacterial infection in an autocrine fashion (Ramirez-Carrozzi et al., 2011; Song et al., 2011; Huang et al., 2015). In human, IL-17C secreted by colonic enteroendocrine and goblet cells plays an important role in the onset of IBD. These IL-17C-mediated effects were amplified by IL-17A (Ramirez-Carrozzi et al., 2011). Here, the ETEC induced IL-17C was mainly secreted by goblet cells. Interestingly, IEC-derived IL-17A preceded IL-17C production and blocking IL-17A abolished IL-17C production. We propose that

during infection small IECs produce IL-17A triggering epithelial IL-17C production, which then drives epithelial barrier fortification in an autocrine manner.

Bacterial adhesion to the intestinal epithelium seems necessary to elicit Th17 responses via epithelial production of serum amyloid A (SAA) (Atarashi et al., 2015; Sano et al., 2015). As ETEC adhesion is required to trigger robust epithelial production of IL-17A and Th17 responses are required to resolve ETEC infections, future work could elucidate if ETEC triggers epithelial SAA to drive Th17 responses.

In summary, our findings reveal that both enterocytes and goblet cells sense the presence of the ETEC-specific enterotoxin STb, triggering their secretion of IL-17A upon ETEC infection and thereby promoting the fortification of the epithelial barrier. Our findings provide further insights in the function of enterocytes and goblet cells during infection and may inform on the development of therapeutics aiming at enhancing the barrier function of the intestinal epithelium.

Chapter 6

General discussion and future perspectives

6.1 Introduction

F4⁺ enterotoxigenic *E. coli* (ETEC) is one of the most common causes of diarrhea in neonatal and recently weaned piglets, resulting in morbidity and mortality. Both F4 fimbriae and enterotoxins play an important role in the pathogenesis of F4⁺ ETEC-associated diarrhea in pigs. The F4 fimbriae mediate the attachment of the bacteria to F4-specific receptors (F4R) present on small intestinal epithelial cells (IECs) (Bakker et al., 1992b; Van den Broeck et al., 1999a). This attachment enables colonization of the small intestine by the bacteria and the release of their enterotoxins, LT and STa/b, which ultimately lead to diarrhea. Immunity to F4⁺ ETEC infection requires F4 fimbriae-specific SIgA, which prevents the adhesion of the bacteria to the intestinal epithelium. Recently, Th17 cells and their production of IL-17A have been implicated in the induction of SIgA directed against gut-dwelling pathogens (Cao et al., 2012; Hirota et al., 2013). Although previous studies have shown that the enterotoxin LT triggered IL-17A secretion by human PBMCs and murine splenocytes, which correlated with increased levels of antigen-specific IgG_{2a} and IgA in mice (Norton et al., 2012; Leach et al., 2012), no clear protective role for Th17 cells during ETEC infection has been identified. In addition, previous work at our lab demonstrated the upregulation of IL-17A mRNA in small intestinal tissues the first 4 hours post F4⁺ ETEC inoculation (Loos et al., 2012). As differentiation and maturation of antigen-specific Th17 cells takes around 3–5 days, the aims of the present study were to investigate the cellular source of this innate IL-17A production as well as to elucidate if Th17 cells are involved during F4⁺ ETEC infection.

6.2 Early molecular warfare between host and ETEC at the epithelium

Within 4 hours after F4⁺ ETEC infection, IL-17A mRNA are observed in the IECs along the villi and in the crypts as well. Although we couldn't specify which type of cells type contribute the early IL-17A mRNA production, at day 4 after F4⁺ ETEC infection

we observed CK18⁺ cells with a goblet cell morphology, which can also produce IL-17A. Thus, we assume the goblet cells are capable of producing IL-17A during the early stage of ETEC infection as well. However, goblet cells are not the only source of early IL-17A production as indicated from the results of FISH and IHC. Probably, enterocytes and other innate lymphocytes also contribute to the IL-17A production after F4⁺ ETEC infection, such as type 3 innate lymphoid cells (ILC3) and gammadelta T ($\gamma\delta$ T) cells). Human ETEC are able to degrade MUC2 and other mucins in the small intestine, thus promoting bacterial colonization of the small intestine and accelerating toxin access to the epithelial surface (Zhou et al., 2012; Kumar et al., 2014; Luo et al., 2014). This process of mucin degradation is mediated by an ETEC autotransporter A (EatA), which are highly homologous to the antigen SepA and commonly detected in porcine O149 ETEC isolates (Boerlin et al., 2005). Thus, disruption of the mucus barrier may also happen during porcine ETEC infection. This intestinal mucus disruption results in a defective mucus barrier with increased permeability that results in inflammation and injury of the intestinal epithelial cells, which may also trigger secondary intestinal infections. The maintenance of the mucus layer is accomplished by specialized secretory enterocytes, such as goblet cells (McGuckin et al., 2011). These cells are the main source of the major mucin MUC2 and other mucins as well as mucus components, such as FCGBP (Kim and Khan, 2013; Pelaseyed et al., 2014). Therefore, it is reasonable to presume that the enterocytes derived IL-17A contributes to the MUC2 replacement and barrier repairment. This is supported by the evidence that F4⁺ ETEC infection induced 1) rapid IL-17A mRNA expression in the epithelial cells and 2) IL-17A can induce the MUC2 production in porcine IECs (IPEC-J2 cells). In addition, IL-17A also enhanced the host defense against ETEC by promoting the pBD-2 mRNA expression in IPEC-J2 cells. Goblet cells, however, seem not only responsible for MUC2 production, but are also capable of delivering small luminal antigen to IgA promoting CD103⁺ dendritic cells in the small intestine (McDole et al., 2012). Interestingly, colocalization of MUC2 and IgA was found in subepithelial cells within the villi of F4⁺ ETEC infected piglets (**Figure 1**). These cells are supposed to be antigen-presenting cells, such as DCs and macrophages, since they can internalize SIgA, which mediates bacterial Ag translocation (Kadaoui et al., 2007).

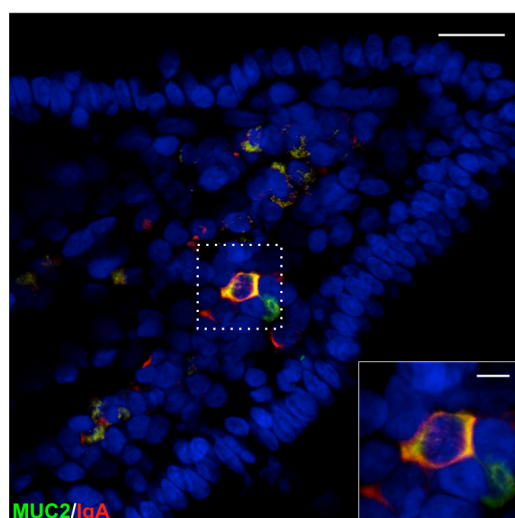


Figure 1 Interaction between DC-like cells and goblet cells. Piglets were infected with F4+ETEC and on day 4 intestinal tissues were sampled. Cryosections were stained with anti-MUC2 (FITC, green) and anti-porcine IgA mAb (Texas Red-X, red). The nuclei were counterstained with Hoechst. Scale bar = 25 μ m.

Previous results demonstrated a fast IL-17A mRNA induction in the small intestinal tissues after F4⁺ ETEC infection (Loos et al., 2012) and indicated STb contributes to this rapid IL-17A response. Here, we further provide evidence that STb induces the production of IL-17A in IECs. As mentioned before, STb binds sulfatide (3'-sulfogalactosyl - ceramide) (Beausoleil et al. 1999; Gonçalves et al. 2008), however, this binding does not alter cGMP or cAMP levels in IECs, but leads to uptake of Ca²⁺ into the IECs (Fujii et al., 1997). Increased cellular Ca²⁺ is capable of eliciting a wide range of cellular responses, such as regulating transcription factor activity and subsequent gene expression (Clapham, 2007). Ca²⁺ first bind to calmodulin composing the Ca²⁺/calmodulin complex, which can be preferentially phosphorylated by calcineurin compared to other phosphatase (Mellström et al., 2008). These processes further result in NFAT dephosphorylation and translocation into the nucleus leading to enhanced IL-17 promoter activity and increased IL-17 expression following T cell receptor (TCR) activation (Liu et al., 2004). Moreover, a NFAT binding site was found which is more downstream compared to the NF- κ B binding site, indicating NFAT may play a more prominent role in inducing IL-17A (Liu et al., 2004). Recently, the increased expression of MUC2 and differentiation of goblet cells was found to be associated with the activation of NFAT in the human intestinal epithelial cell line Caco-2 (Zhou et al., 2014). Thus, after ETEC infection, STb-mediated IL-17A strengthened the barrier by increasing the production of MUC2 expression in IECs, probably associated with NFAT signaling. However, further studies are needed to clarify possible

mechanisms of STb in the promotion of IL-17A.

As goblet cells, enteroendocrine cells and colon epithelial cells were found to express IL-17C (Friedrich et al., 2015; Ramirez-Carrozzi et al., 2011), we also detected the protein and mRNA expression of this cytokine in the intestinal tissues and IPEC-J2 cells after F4⁺ ETEC infection. Notably, IL-17C was also dramatically induced after F4⁺ ETEC infection and mainly localized in goblet cells. Besides goblet cells, we also detected a small number of IL-17C producing cells are presumably enteroendocrine cells (IL-17C⁺CgA⁺ phenotype), indicating an interaction between the neuroendocrine system and immune system during ETEC infection. Indeed, enteroendocrine cells can sense bacterial antigens through TLRs and are involved in neutralizing intestinal bacteria by releasing chemokines and defensins (Palazzo et al., 2007). ETEC infection may also increase the epithelial permeability by either reducing the expression (Gao et al., 2013) or redistributing tight junction proteins, such as Claudin-1 (Ngendahayo Mukiza and Dubreuil, 2013; Nassour and Dubreuil, 2014), thus allowing the passage of electrolytes and water through the paracellular space and trigger diarrhea. In this context, IL-17C probably was required to re-establish the epithelial homeostasis during F4⁺ ETEC infection since this cytokine regulates the production of antimicrobial peptides, tight junction proteins and defensins (Ramirez-Carrozzi et al., 2011; Reynolds et al., 2012; Kusagaya et al., 2014). Although the IL-17C response appears rapidly in epithelial cells after *E. coli* and inflammatory stimuli (Ramirez-Carrozzi et al., 2011), the mechanism for fast regulation of this cytokine remains elusive. TLR2, 3 and 5, which recognize bacterial lipopeptides, polyinosinic-polycytidylic acid (polyI:C) and flagellin respectively, have been reported to be involved in IL-17C induction (Ramirez-Carrozzi et al., 2011; Im et al., 2012; Kusagaya et al., 2014). In our study, the IL-17C induction after ETEC infection can be attributed (at least partially) to STb associated IL-17A production.

In addition to the IL-17 cytokines, mRNA expression of IL-1 β and IL-8 were also upregulated in intestinal tissues 4 h after F4⁺ ETEC perfusion (Loos et al., 2012). These transcripts are probably derived from IECs as the upregulation of these two transcripts were also found in IPEC-J2 cells within 2 h after F4⁺ ETEC stimulation. An elevation in these pro-inflammatory cytokines seems necessary for the clearance of

bacteria by facilitating the activation of mast cells and recruitment of neutrophils (McLamb et al., 2013).

6.3 Adaptive immunity during late period of F4⁺ ETEC infection

Four days after F4⁺ ETEC infection, IL-17A and other Th17-related genes were upregulated in either the intestinal tissues or in the PBMCs, indicating that Th17 cells may have been induced at this timepoint. Meanwhile, influx of CD3⁺ T cells and CD172a⁺ cells was observed in the ileum, indicating the participation of T cell responses and other myeloid cells, such as dendritic cells and macrophages in the later steps of infection. Most of these T cells produced IL-17A and assumed Th17 cells since Th17-related cytokines were also induced after F4⁺ ETEC infection in both the intestine and blood.

Th17 cells have been connected with B-cell proliferation, isotype class switching as well as SIgA production (Jaffar et al., 2009; Mitsdoerffer et al., 2010; Barbosa et al., 2011; Cao et al., 2012; Hirota et al., 2013; Milpied and McHeyzer-Williams, 2013). In this context, Th17 cells/ IL-17A also direct SIgA production after F4⁺ ETEC infection since significant higher levels of IgA secretory component (SC) were observed in the ileum of infected pigs. This finding is correlated with a higher induction of AID, which is required for the antibody class switching to IgA, and an enhanced expression of plgR, which enables transport of dimeric IgA to the apical surface of epithelial cells. The secreted antigen-specific SIgA confers protection against ETEC infection by preventing the binding of bacteria to the epithelium and by neutralizing the enterotoxins (Van den Broeck et al., 1999a,b; Verdonck et al., 2002). IL-17F mRNA also increased after F4⁺ ETEC infection, probably also coming from Th17 cells and contributing to the host defense (Ishigame et al., 2009; Cho et al., 2010; Aujla et al., 2008). The elevated Th17-related cytokines during F4⁺ ETEC infection, for example IL-21 and IL-22, probably also promotes intestinal IgA response and barrier function against the bacteria infection (Cao et al., 2015; Sonnenberg et al., 2011).

In contrast to Th17, Th1 cells may not be essential for the host defense against F4⁺ ETEC infection, since only a small increased IFN- γ mRNA expression was observed in the PBMCs later during ETEC infection. However, Tregs are probably

required to limit the inflammatory response and to avoid immunopathology due to an overwhelming Th17 immunity during F4⁺ ETEC infection (Käser et al., 2008; Ouyang et al., 2011). It is worth noting that after F4⁺ ETEC infection, IL-17B mRNA was increased in the intestinal tissues but downregulated in the PBMCs. Moreover, its specific receptor IL-17RB was highly induced in the IPEC-J2 cells after F4⁺ ETEC infection, indicating they probably required for the immune regulation in the gut. Recently, colon epithelial cells derived IL-17B were found to prohibit IL-17E-mediated inflammatory responses by binding to their receptor IL-17RB, thus playing a beneficial role in acute colitis and *Citrobacter rodentium* infection (Reynolds et al., 2015). We didn't detected IL-17E mRNA in the porcine IPEC-J2 cells after ETEC infection (data not shown), so further work is needed to understand the function of these two cytokines during bacterial infection.

6.4 Main conclusions

CD4⁺ T helper cells are important mediators of adaptive immune responses. It is becoming clear that Th17 cells and their hallmark cytokine IL-17A is beneficial against dozens of pathogens, especially at mucosal sites. Thus, in the first study we investigated if Th17 cells are involved in the induction of protective immunity to F4⁺ ETEC infection. As IECs are the first cells encountering ETEC and elevated IL-17A transcripts were found in the intestinal tissues 4 h upon ETEC infection, in the second study we further identified the cellular source of this early IL-17A mRNA and its potential roles in innate defense. In this section, however, I will present the infection and immune response in a chronological order.

As shown in **Figure 2**, both innate and adaptive immune responses are evoked to clear F4⁺ ETEC infection. For the hosts, infection results in disruption of the epithelium and tissue damage by the enterotoxins and inflammatory injury. IECs are the first cells to encounter these pathogens and initiate the immune response in the intestine after F4⁺ ETEC infection. In the early steps of infection, enterocytes (probably goblet cells also)-derived IL-17A are necessary for the promotion of MUC-2, pBD-2, which are essential for the maintenance of the epithelium and host defense. Besides, cytokines, including IL-8, IL-1 β and TNF- α , and chemokine CCL20 are also triggered fast in the

enterocytes probably also needed against bacterial infection and/or control of inflammation. For example, CCL20 can recruit DCs from blood to the LP of intestine and to initiate an adaptive response.

In the later period of infection, local naïve T cells or resident pre-Th17 cells are activated and differentiate into Th17 cells at the inductive sites in either PP or MLN under the direction of certain cytokines. In the same microenvironment, Th17 cells interact with naïve B cells and responsible for the class switching of IgA⁺ B cells with the help of cytokines including IL-17A, IL-21 and AID. Then, these primed Th17 cells and IgA⁺ B cells migrate through lymphatics to the blood circulation and, finally home back to the lamina propria via high endothelial venules (HEV). Ag-specific DCs interact with these activated Th17 cells, further regulating high affinity IgA⁺ production. Meanwhile, IL-17A coming from goblet cells and/or Th17 cells regulates the pIgR production and subsequent IgA secretion, which is required for bacterial neutralization.

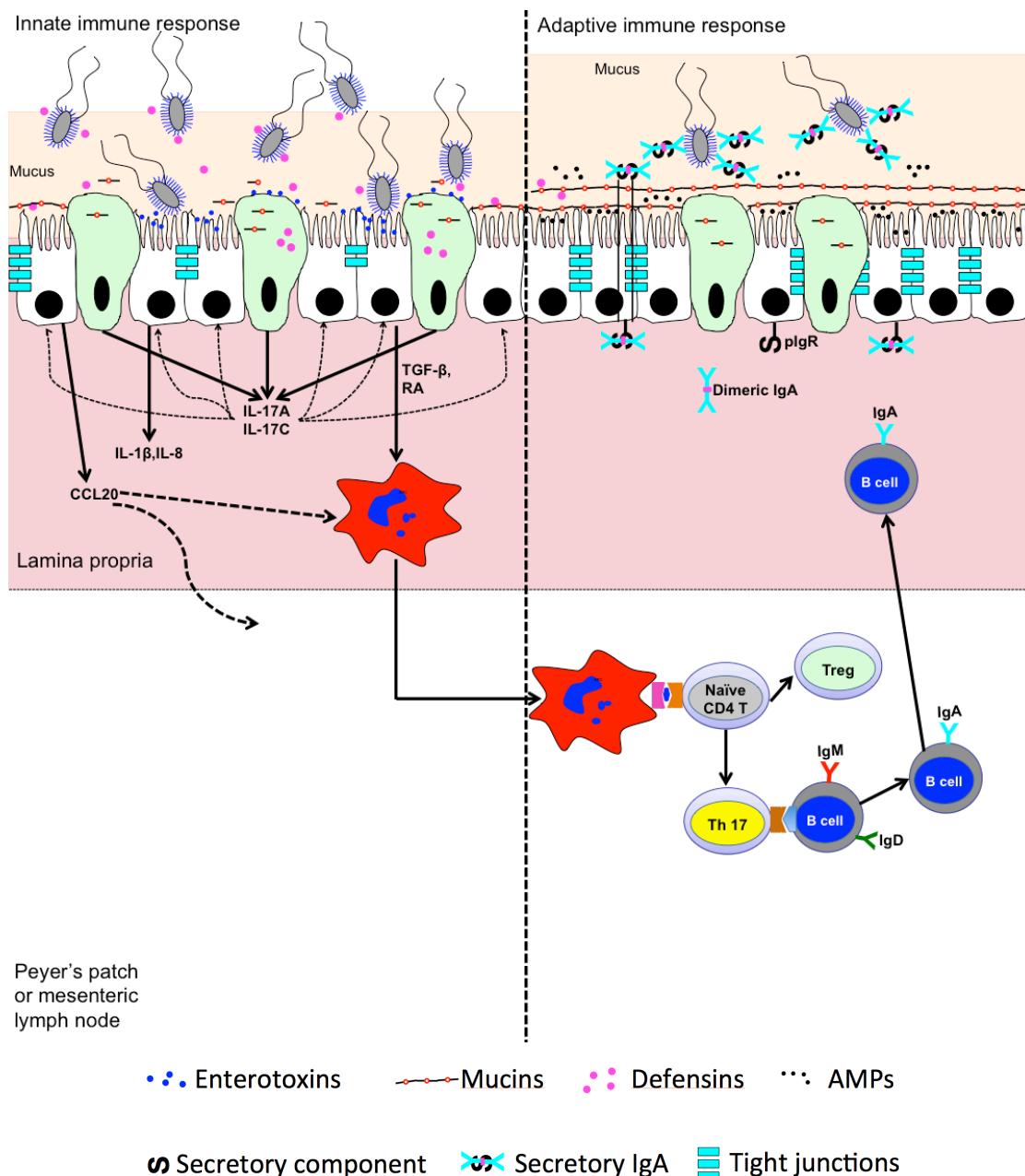


Figure 2. Immune responses after ETEC infection. After binding to the intestinal epithelial cells (IECs), ETEC starts secreting enterotoxins, which can bind to certain receptors on the IECs and disrupt the water and electrolyte balance. Meanwhile, enterotoxins such as STb also triggers cytokines induction in the IECs, such as IL-17A, IL-1 β , IL-8 and CCL20. IL-17A and/or IL-17C can immediately bind to the neighbouring IECs and may contribute for the MUC2 replacement and the production of defensins, such as beta-defensin-2. Other cytokines may be involved in the recruitment of other immune cells to the lamina propria to clear the bacterial infections, for example neutrophils and dendritic cells. ETEC or its virulence factors may also be sensed by APCs (e.g. dendritic cells) and activate naïve CD4⁺ T cells, which mainly differentiate into Th17 cells. These Th17 cells and their cytokines direct the IgA class switching of B cells, resulting in higher affinity and production of secretory IgA.

6.5 Limitations and future perspectives

Although Th17 cells seems to be the important cellular source of IL-17A 4 days after F4⁺ ETEC infection, other T cells can not be excluded. For example, gammadelta T cells ($\gamma\delta$ T cells) and type 3 innate lymphoid cells (ILC3) also have the ability to produce IL-17A. So, it would be very interesting to identify if these innate immune cells are also involved during ETEC infection. The noninvasive F4⁺ETEC adheres and colonizes the small intestine by using the F4 fimbriae. This adhesion property decides these bacteria generally elicit Th17 cells but not Th1 cells (Atarashi et al., 2015). Thus, piglets immunized with F4 fimbriae also induced robust Th17 instead of Th1 response. Induction of Th17 cells seems also depends on the integrity of epithelial layer during bacterial infection, as intact epithelial layer induced prominent Th17 but not Th1 response and vice versa (Atarashi et al., 2015). Thus, the early IL-17A production in the IECs may also contribute for the Th17 cell development, as this cytokine participated in mucin replacement and barrier repairmen after ETEC infection. Robust IL-17A and IL-17C are induced by enterocytes and by goblet cells after ETEC infection and their expression seems to depend on the presence of STb. However, further research is needed to elucidate how STb regulates this IL-17A production. Moreover, the contribution of other toxins to IL-17A induction in the early stage of ETEC infection should also be investigated, since a synergistic effect of the enterotoxins LT and/or STa on the IL-17 mRNA production was also observed.

F4-specific IgA and IgG is elicited as early as 4 days after F4⁺ETEC infection, while F18-specific antibodies were hardly detected in the intestinal tissues at the same timepoint after F18⁺ ETEC infection (Verdonck et al., 2002). This F4-specific IgA response is probably linked to the induction of Th17 cells and their cytokines (Luo et al., 2015). Considering the important role of Th17 cells in IgA induction, more detailed research should be performed to better understanding the relationship between Th17 cells and IgA regulation in the intestine in pigs after ETEC infection. For example, whether the defects in IgA production is coming from the incapable of Th17 induction after F18⁺ ETEC infection. Recently, IECs-derived serum amyloid A (SAA) was found to contribute to IL-17A production in Th17 cells in mice (Atarashi et al., 2015; Sano et al., 2015). Serum amyloid A (SAA) is a plasma protein, which is typically induced in

response to inflammation or injury as a part of the acute-phase response (Uhlir and Whitehead, 1999). Recently, SAA has been found to be involved in the binding and transport of retinol during bacterial infection (Derebe et al., 2014). The metabolite of retinol, retinoic acid (RA), influences the initiation, homing capacity and polarity of CD4⁺ T cell responses. For example, RA favors Foxp3 expression and the development of Tregs by enhancing TGF- β expression (Elias et al., 2008), even in the presence of IL-6 or IL-21 (Xiao et al., 2008). Thus, these findings lead to a new question: is the Th17-inducing effect of SAA dependent on its retinol binding, resulting in a low RA activity at the microenvironment? Besides, SAA mediated IL-1 β production through NACHT, LRR and PYD domains-containing protein 3 (NLRp3) inflammasome activation is found in several cells, like keratinocytes, dendritic cells and macrophages (Allam et al., 2011; Yu et al., 2015b). Notably, SAA expression in keratinocytes can be stimulated by IL-17A supplementation (Yu et al., 2015). Since IL-1 β also contributes to the initiation of the Th17 response, SAA may be a messenger in the cross talk between the Th17 and IL-1 β pathways. It will be interesting, in future studies, to evaluate SAA production in porcine IECs or intestinal tissues and characterize its potential role in host defense and the signaling mechanism required for the Th17 response during ETEC infection.

Notably, we also detected the mRNA expression of other IL-17 family members (IL-17B and IL-17F) in the intestine tissues of pigs after F4⁺ ETEC infection. Moreover, receptors for all of these IL-17 cytokines were detected in the porcine small intestinal epithelial cell line IPEC-J2, and significantly increased after ETEC stimulation. These findings suggested these cytokines also required against F4⁺ ETEC infection. Identifying the cellular source of these cytokines and their regulation mechanisms could provide potential insights for promoting mucosal immune responses during ETEC infection in pigs. Since IPEC-J2 cells were isolated from neonatal piglet mid-jejunum, these cells may display pluripotency and could have the potential to differentiate into MUC2⁺ goblet cells and CgA⁺ enteroendocrine cells. In addition, transepithelial resistances (TER) and transport properties of IPEC-J2 can be also modified under certain culture conditions (Zakrzewski et al., 2013). These findings highlight that the influence of culture conditions on cell structure and subsequent

functionality should be considered.

ETEC still affects many children and travelers to areas where ETEC is endemic. Despite many efforts, no ETEC vaccines are currently available. A significant challenge to successful vaccine development is the poor understanding of the intestinal innate and adaptive immune responses upon infection. Since piglets are also susceptible to ETEC infections, they represent a unique model to study host-pathogen (ETEC) interactions. The present results indicated Th17 cells participated in the protective immunity to ETEC infection in piglets and that these Th17 responses are also in part induced by oral immunization with F4 fimbriae. We also found out that STb can induce early IL-17A production in enterocytes and goblet cells, which further controls the production of IL-17C, mucins, antimicrobial peptides and plgR by these cells upon ETEC infection. F4 fimbriae are a potent immunogen, which can induce F4-specific SIgA at the mucosal sites. STb on the other hand seems to regulate the innate immune response, but is poorly immunogenic because of its small molecular size. LT, however, is a mucosal adjuvant and found to induce IL-17A production as well as IgG and IgA production in human and mice. Thus, a future porcine-specific ETEC vaccine should at least combine F4 fimbriae, STb and LT, which can induce rapid innate defense and efficient F4-specific SIgA response to fully protect piglets against F4⁺ ETEC infection.

Summary

In neonatal and recently weaned pigs, ETEC-associated diarrhea is a major cause of illness and mortality, leading to great economic losses in the swine production industry worldwide. F4⁺ ETEC is one of the most prevalent porcine ETEC strains and plays an important role in the development of postweaning disease. To effectively protect piglets against F4⁺ ETEC infection, fast ETEC-specific SIgA responses are required. This can be achieved by oral immunization of piglets with F4 fimbriae, as this triggers protective F4 fimbriae-specific SIgA responses. Recently, Th17 cells and their cytokines have been identified to play a cardinal role in host defense against several mucosal pathogens by enhancing the barrier function of the intestinal epithelium as well as promoting SIgA production. However, if Th17 cells can trigger SIgA responses and thus confer protection to F4⁺ ETEC infection in piglets is unknown. In addition, previous work showed elevated IL-17 mRNA transcripts during early ETEC infection in the porcine small intestinal tissues, but the cellular source of this innate IL-17A remains unclear.

Chapter 1 reviews the literature on the pathogenesis of ETEC in pigs with a focus on the main virulence factors of the most prevalent porcine ETEC strains. In addition, several strategies to prevent ETEC infection in neonatal and postweaning piglets are reviewed.

Chapter 2 summarizes the current knowledge of the innate and adaptive immune response upon ETEC infection in pigs. Special attention is given to intestinal epithelial cells, as these cells provide a first line defense against intestinal pathogens and are pivotal in informing the mucosal immune system on the encountered luminal threats. Innate immune cells, T help cells and their roles in host defense against several gut extracellular pathogens are also described in this chapter. As Th17 cells exert their function mainly through IL-17A and related cytokines, an overview of the current knowledge of the function of the IL-17 cytokine family is also given.

Chapter 3 describes the aims of this doctoral thesis, which are to elucidate if Th17 cells are involved in the induction of protective immunity to F4⁺ ETEC and identify the cellular source of ETEC-induced IL-17A mRNA upon early infection.

In **Chapter 4**, we sought to determine if Th17 cells play a role in the induction of protective immunity during ETEC infection. Therefore, we assayed the immune response in piglets after F4⁺ ETEC infection and oral immunization with F4 fimbriae. By analyzing the mRNA expression profile of key cytokines and transcription factors involved in the differentiation of Th1, Th2, Th17, and Tregs in both PBMCs and intestinal tissues, we observed an increased expression of the Th17-related genes IL-17A, IL-17F, IL-21, IL-22, IL-23p19 and ROR γ t. A high expression of IL-17A mRNA in the ileal Peyer's patches of F4⁺ ETEC infected piglets was detected and this IL-17A was most likely produced by Th17 cells as an influx of CD3⁺IL-17A⁺ cells was observed in the crypts and villi of F4⁺ ETEC infected piglets as compared to control pigs. These responses upon F4⁺ ETEC infection were largely mimicked by oral immunization of piglets with F4 fimbriae. Interestingly, we also observed an elevated IL-17B response upon F4 fimbriae immunization, for the first time linking IL-17B to intestinal immunity. Altogether, these findings indicate that Th17 cells and IL-17A play crucial roles in the protection of piglets against F4⁺ ETEC infection.

In **Chapter 5**, we aimed to identify the cellular source of the early small intestinal IL-17A mRNA production upon ETEC infection and its potential roles in immunity. Using FISH and confocal microscopy, we further confirmed these elevated IL-17A transcripts upon ETEC infection in the small intestine. Surprisingly, the IL-17A transcripts were localized in small intestinal epithelial cells. At a later timepoint during infection, we demonstrated that enterocytes and goblet cells are the main source of IL-17A along the villi and in the crypts, implying that these cells probably contribute to the early IL-17A mRNA production. Likewise, we also found that IL-17C is induced after F4⁺ ETEC infection and is mainly secreted by goblet cells and to a lesser extent by enteroendocrine cells. Using enterotoxin deficient ETEC mutant strains, bacterial culture SN of these strains and purified STb, we showed that this early IL-17A and IL-17C mRNA production by epithelial cells after ETEC infection was dependent on the presence of the enterotoxin STb. The induced IL-17A immediately bound to its receptor on neighboring epithelial cells and regulated their production of pIgR, pBD-2, MUC2 and IL-17C, which are all involved in the fortification of the intestinal epithelial barrier. Altogether, these data clearly show that small intestinal epithelial cells, such as

enterocytes and goblet cells, produce IL-17A upon detection of the ETEC enterotoxin STb to enhance the epithelial barrier function.

In **Chapter 6**, the general discussion, the future perspectives and the potential applications of our findings are presented. The interactions between ETEC and the host in the gut are highly complex and far from being completely understood. Upon sensing ETEC enterotoxins, intestinal epithelial cells and innate immune cells are activated to clear ETEC by producing IL-17 cytokines, AMPs and mucins. Later on during infection, Th17 cells take over. It would be interesting to figure out the IL-17A/C signaling mechanisms in intestinal epithelial cells. Future studies on the mechanism of intestinal Th17 cell induction will be useful to enhance the efficacy of mucosal vaccines to prevent ETEC infections.

Samenvatting

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In neonatale en recent gespeende biggen vormt ETEC-geassocieerde diarree een belangrijke oorzaak van ziekte en sterft, wat resulteert in zware economische verliezen voor de wereldwijde varkensindustrie. F4⁺ ETEC is één van de meest prevalentie varken ETEC stammen en speelt een belangrijke rol in de ontwikkeling van speendiarree. Om biggen effectief te beschermen tegen F4⁺ ETEC infecties, zijn snelle ETEC-specifieke SIgA responsen vereist. Dit kan bekomen worden door orale immunisatie van biggen met F4 fimbriae, aangezien dit leidt tot de opwekking van beschermende F4 fimbriae-specifieke SIgA responsen. Recent werd aangetoond dat Th17 cellen en hun cytokines een belangrijke rol spelen in de bescherming van de gastheer tegen verschillende mucosale pathogenen, aangezien deze cellen zowel de epitheliale barrière versterken als de productie van SIgA bevorderen. Maar het is nog steeds niet geweten als Th17 cellen in staat zijn om SIgA responsen op te wekken en zo biggen kunnen beschermen tegen F4⁺ ETEC infecties. Daarnaast heeft recent onderzoek aangetoond dat vroeg tijdens de ETEC infectie verhoogde IL-17A mRNA transcripts aanwezig zijn in de dunne darm weefsels, maar de cellulaire bron van dit aangeboren IL-17A is onbekend.

Hoofdstuk 1 geeft een overzicht van de pathogenese van ETEC in biggen met een focus op de belangrijkste virulentiefactoren van de meest prevalentie varken ETEC stammen. Bovendien wordt er een overzicht gegeven van de verschillende strategieën om ETEC infecties in neonatale en recent gespeende biggen te voorkomen.

Hoofdstuk 2 vat de huidige kennis over de aangeboren en adaptieve immuunrespons na ETEC infectie in biggen samen. Er wordt ook aandacht gegeven aan de intestinale epitheliale cellen, aangezien deze cellen een eerste lijn verdediging vormen tegen intestinale pathogenen en eveneens belangrijk zijn om informatie over lumenale gevaren door te geven aan het mucosaal immuunsysteem. Aangezien Th17 cellen een belangrijke rol spelen in de inductie van antigeen-specifieke SIgA, wordt in dit hoofdstuk ook de differentiatie van deze cellen en hun rol in de bescherming van de gastheer tegen extracellulaire pathogenen besproken. Aangezien Th17 cellen hun functie hoofdzakelijk uitoefenen via IL-17A en gerelateerde cytokines, wordt eveneens een overzicht gegeven van de huidige kennis over de functie van de IL-17 cytokine

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familie.

In **Hoofdstuk 3** worden de doelstellingen van deze doctoral thesis beschreven. Het doel van deze thesis is enerzijds om na te gaan als Th17 cellen betrokken zijn in de opwekking van een beschermende immuniteit tegen F4⁺ ETEC infecties in biggen en anderzijds om de cellulaire bron van de verhoogde IL-17A mRNA productie tijdens de vroege fase van een ETEC infectie te identificeren.

In **Hoofdstuk 4** werd de immuunrespons in biggen onderzocht na F4⁺ ETEC infectie en orale immunisatie met F4 fimbriae. Door het mRNA expressie profiel van sleutel cytokines en transcriptiefactoren, die betrokken zijn in de differentiatie van Th1, Th2, Th17 en Tregs, in zowel de PBMCs als intestinale weefsels te onderzoeken, werd een verhoogde mRNA expressie van de Th17-gerelateerde genen IL-17A, IL-17F, IL-21, IL-22, IL-23p19 en ROR γ t gedetecteerd. Er werd een hoge IL-17A mRNA expressie gedetecteerd in de ileale Peyerse platen van F4⁺ ETEC-geïnfecteerde biggen. Dit IL-17A werd vermoedelijk geproduceerd door Th17 cellen, aangezien een sterke influx van CD3⁺IL-17A⁺ cellen werd geobserveerd in de crypten en villi van F4⁺ ETEC geïnfecteerde dieren in vergelijking met controle dieren. Deze responsen na F4⁺ ETEC infectie konden grotendeels nagebootst worden door orale immunisatie van de biggen met F4 fimbriae. Bovendien werd een verhoogde IL-17B expressie waargenomen na orale immunisatie, waarbij voor de eerste keer IL-17B geassocieerd kan worden met intestinale immuniteit. Samenvattend wijzen deze bevindingen op een belangrijke rol van Th17 cellen en IL-17A in de bescherming van biggen tegen F4⁺ ETEC infecties.

In **Hoofdstuk 5** werd geprobeerd om de cellulaire bron van de vroege IL-17A mRNA productie in de dunne darm na ETEC infectie te identificeren en de mogelijke rollen in immuniteit. Door gebruik te maken van FISH en confocale microscopie waren we in staat om deze verhoogde IL-17A transcripts na ETEC infectie in de dunne darm te bevestigen. Deze IL-17A transcripts lokaliseerden verrassend genoeg in de epitheelcellen van de dunne darm. Op een later tijdstip tijdens de infectie werd aangetoond dat enterocyten en slijmbekercellen de voornaamste bron zijn van dit IL-17A, wat suggereert dat deze celtypen waarschijnlijk ook IL-17A mRNA produceren tijdens de vroege fase van de infectie. Behalve IL-17A werd ook IL-17C productie opgewekt na ETEC infectie. IL-17C werd voornamelijk geproduceerd door

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slijmbekercellen en in mindere mate door enteroendocriene cellen. Door gebruik te maken van enterotoxine deletie mutanten, bacterieel cultuur SN van deze stammen en opgezuiverd STb, werd aangetoond dat de productie van IL-17A en IL-17C door epitheelcellen na ETEC infectie afhankelijk was van de aanwezigheid van het STb enterotoxine. Het opgewekte IL-17A bond meteen aan zijn receptor op naburige epitheelcellen en reguleerde hun productie van plgR, pBD-2, MUC2 en IL-17C. Deze eiwitten zijn allemaal betrokken in de versterking van de intestinale epitheliale barrière. Deze bevindingen tonen duidelijk aan dat epitheelcellen in de dunne darm, zoals enterocyten en slijmbekercellen, IL-17A produceren na detectie van het ETEC-specifieke enterotoxine STb om de functie van de epithelial barrière te versterken.

Hoofdstuk 6 geeft de algemene discussie weer en stelt de toekomstperspectieven en de potentiële toepassingen van de bevindingen voor. De interacties tussen ETEC en de gastheer zijn zeer complex en zijn nog niet volledig opgehelderd. Na het detecteren van ETEC enterotoxines, starten intestinale epitheelcellen en aangeboren immuuncellen een moleculaire oorlog tegen ETEC door de productie van IL-17 cytokines, AMPs en mucines. Later tijdens de infecties, nemen Th17 cellen dit over. Het zou interessant zijn om de IL-17A/C signaal mechanismen in intestinale epitheelcellen te ontrafelen. Bovendien zou het nuttig zijn om in de toekomst het mechanisme van intestinale Th17 cel differentiatie op te helderen om zo de effectiviteit van mucosale vaccins gericht tegen ETEC te verhogen.

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Curriculum Vitae

Yu Luo was born on October 15th, 1986 in Qianjiang, China. In 2008, he earned the Bachelor of Science in Agriculture from Huazhong Agricultural University, Wuhan, China. Yu worked in the National Key Laboratory of Agricultural Microbiology as an undergraduate from 2007 until 2008, on a research project identifying a potential protective antigen against *Haemophilus parasuis*. From 2008 until 2011, he worked in the National Key Laboratory of Agricultural Microbiology with a research focused on the role of prostaglandin E₂ on the immune cells after infection with *Mycobacterium bovis*. In 2011, he earned the Master degree in Veterinary Medicine at Huazhong Agricultural University. In September 2011, he started his PhD research entitled at Faculty of Veterinary Medicine, Ghent University. This project was sponsored by Chinese Scholarship Council (CSC) and the Special Research Fund of Ghent University (cofunding of the CSC Grant).

Yu Luo is the author and co-author of several publications in peer-reviewed international journals and he participated in several international conferences.

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1. Publications in peer-reviewed international journals

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2. Abstracts and posters presented at international conferences

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Devriendt B, Baert K, **Luo Y**, Cox E. 2014. ETEC colonisation factors abrogate the antigen presentation function of porcine intestinal mononuclear phagocytes. *The 13th international symposium on dendritic cells (DC2014)*, poster presentation, 14-18 sept, Tours, France.

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Devriendt B, Baert K, **Luo Y**, Cox E. 2012. ETEC colonisation factors disrupt the antigen presenting capacity of porcine intestinal dendritic cells. In *CFCD, Congrès Annual, Résumés*, ed. Bert Devriendt. Club Francophone des Cellules Dendritiques (CFCD).

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Gent, 28th January 2016

Yu Luo

